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## ERRATUM

Volume XXXIII, No 1.

page 112, last line: *for* sequence *d, f, pH 9, sc, ae, .*  
*read* sequence *d, f, pH 9, sc, ae, f.*

Vol. XXXIII, No. 4

page 418, line 81 *for* 20 m $\mu$  *read* 220 m $\mu$

Volume XXXIII, No. 5

page 745, line 22 *for* 2, 4 and 6 C atoms  
*read* 3, 5 and 7 C atoms

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page 788, line 4 *for*  $c = k \cdot \frac{s-x}{x} \cdot \frac{s}{100}$   
*read*  $c = k \cdot \frac{x}{s-x} \cdot \frac{s}{100}$

page 840 *for* Fig. 3 *read* Fig. 6

page 845 *for* Fig. 6 *read* Fig. 3



# I. THE EFFECT OF ULTRAVIOLET RADIATION AND OF SOFT X-RAYS ON THE SEDIMENTATION BEHAVIOUR AND LIGHT ABSORPTION OF PURIFIED HUMAN SERUM ALBUMIN

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PROTEIN solutions are sensitive to ultraviolet light, and are coagulated by irradiation at the isoelectric point.<sup>1</sup> Coagulation can be prevented by irradiation either at reduced temperatures (0–4°) [Bovic, 1913] or in the presence of small amounts of acid or alkali (vide Arnow [1936] for references), but it then occurs if the temperature is raised, or the pH brought to the isoelectric point. It appears that coagulation of proteins by u.v. light takes place in at least two stages, a photochemical reaction independent of temperature, and an aggregation of the molecules attacked, a reaction of comparatively high temperature coefficient [Rajewsky, 1929; 1930; Clark, 1935]. The photochemical reaction causes increased light absorption [Spiegel-Adolf & Krumpel, 1927] and cleavage of the molecule, perhaps with the liberation of aromatic amino-acids [Mitchell, 1936].

Pedersen [1931], from preliminary experiments, reported that when serum albumin in solution at pH 3.5 was irradiated by u.v. light, the solution remained clear, the sedimentation constant doubled (indicating aggregation) and the diffusion constant showed a strong drift (indicating heterogeneity).

Previous investigations have not produced much information concerning the state of aggregation of the denatured protein molecules remaining in solution, either before or after the production of a precipitate, or concerning the effects of the substances causing the increased light absorption in irradiated protein solutions, or of pH upon the course of the reactions which follow the initial photochemical reaction. The ultracentrifugal method of analysis is suited to the exploration of a protein system after denaturation since both cleavage and aggregation of the protein molecules will be revealed on the sedimentation diagrams either as distortion of the curves or as separate maxima.

In view of the above, and of the importance of actinotherapy in modern curative medicine, an ultracentrifugal and light absorption study has been made of the effects of u.v. and soft X-ray irradiations on solutions of purified human serum albumin.

## EXPERIMENTAL

Three series of experiments were made: one near the isoelectric point, at pH 5.4, where the irradiation, performed at 30°, caused ultimate coagulation of the protein, and two at pH 7.4 (normal blood pH), one at 30° and the other at 0°

<sup>1</sup> Arnow [1936] has reviewed the literature on the effects of irradiation on proteins and amino-acids.

where all the solutions remained clear. In each series, equal volumes of protein solution were irradiated in a rotating tube for various periods of time, and ultracentrifuged under identical conditions after as nearly as possible the same lapse of time. Experiment showed that rotation without irradiation did not cause surface denaturation. Some stock albumin solution was ultracentrifuged at the beginning and the end of each series to make certain that the solutions were stable.

*Preparation of material.* Fasting blood, sufficient for Series A and B, was drawn from a healthy subject into sterile centrifuge tubes and the resulting serum heated to 56° for 25 min. for preservative purposes. The heating of serum to 56° for as long as 2½ hr. has been found by McFarlane [1935, 2] to have no detectable effect upon its sedimentation diagram. The globulins were separated by half-saturation with  $(\text{NH}_4)_2\text{SO}_4$  according to the directions of Adair & Taylor [1935] and an unsuccessful attempt was made to repeat their preparation of crystalline human serum albumin. When only tiny globules were obtained, the albumin was purified instead by precipitation three times with  $(\text{NH}_4)_2\text{SO}_4$  to saturation. It was dialysed for 30 hr. against distilled water, electro dialysed for 16 hr. and then stored in sterile bottles at 3°. Micro-Kjeldahl N analyses were made using the Parnas & Wagner [1921] distillation apparatus, and the N content converted into concentration of protein by the factor 6.25.

For Series A, 2 ml. stock solution were diluted immediately before irradiation with 0.7 ml. distilled water and 0.3 ml. 10% NaCl solution, giving an albumin concentration of 0.81 g./100 ml.; sterile technique was followed as far as possible.<sup>1</sup> At the end of the series, a solution so prepared gave a pH of 5.44 (glass electrode).

For Series B, 20 ml. stock solution were diluted with 10 ml. of buffer solution containing 84.1 ml. *M*/5  $\text{Na}_2\text{HPO}_4$  and 15.9 ml. *M*/5  $\text{KH}_2\text{PO}_4$  per 100 ml.: the pH (glass electrode) was 7.39. 3 ml. samples were withdrawn for each irradiation experiment.

After the completion of these experiments, a paper by Tiselius [1937] appeared which showed that in serum, heated at 56° for even 10 min., the albumin boundary suffers a large reduction in electrophoretic mobility and migrates near the  $\beta$  globulin; this may indicate incipient denaturation. The albumin used in Series A and B had been prepared from serum heated at 56° for 25 min.: consequently the same change in mobility had probably taken place. Our results and the observations of McFarlane [1935, 2] indicated that this change did not affect the sedimentation diagram, but the possibility remained that the heated material might be more susceptible to irradiation, or might react differently.

For this reason a new stock solution of albumin (for Series C) was made from further blood from the same human; all operations were performed at 0–3°. The globulins were removed by 57% saturation with  $(\text{NH}_4)_2\text{SO}_4$ , which was necessary to prevent precipitation during subsequent dialysis. The albumin was precipitated 3 times by saturation with  $(\text{NH}_4)_2\text{SO}_4$ , dialysed 4 days, electro dialysed for 50 hr. between 0 and 5° and diluted with phosphate buffer to the same protein and buffer concentrations as for Series B; the pH (glass electrode) was 7.40. This stock solution was frozen for storage to prevent bacterial action, Tiselius [1937] having reported that freezing does not affect the electrophoretic behaviour of serum proteins.

During the experiments in Series C, it was observed that to bring about thorough mixing of the protein-rich layer formed at the bottom of the flask on thawing, more vigorous agitation was necessary than the careful swirling which had purposely been employed to prevent foaming. A number of the samples withdrawn (by pipette, from the bottom of the flask) were more concentrated

<sup>1</sup> Spiegel-Adolf & Pollaczek [1929] found delayed coagulation in bacterially infected solutions.

than 0.81 % protein (see initial areas, Table I, which are proportional to concentrations of centrifugible protein). The concentration differences were not great enough to influence the sedimentation constant or spreading coefficient, which are independent of this factor at low concentration, but did affect all values which are concentration-dependent (curve area, height of sedimentation maximum, extinction coefficient). Analysis showed that the samples irradiated 1 and 2 hr. in Series C contained 0.814 and 0.819 % albumin respectively, so that for these comparison with Series A and B is permissible for all values. The solution used for determination of the sedimentation velocity of the untreated material was about 4 % too concentrated (see initial areas, Table I; no solution available for micro-Kjeldahl analysis). For the relationship shown in Fig. 2, the measured height of the sedimentation maximum for this solution was therefore multiplied by the ratio of the initial areas, 0.0046/0.0048.

*Radiation.* The actual conditions of irradiation differed somewhat in the three series of experiments, but remained constant during each series. Directly after irradiation, 0.5 ml. of the solution was removed for the immediate determination of its u.v. absorption spectrum; the remainder was placed in a small sterile bottle, stored at 3° overnight and ultracentrifuged the next morning.

*Series A.* A Uviarc, with a reflector but without a shield in front of the lamp, was the source of u.v. light. The solution was contained in a quartz tube (12.5 cm. long, outer diameter 1.3 cm., inner diameter 1.05 cm.) stoppered with a paraffined cork and placed parallel to the arc, with its axis horizontal, at a distance of 20 cm. The tube was rotated about its axis at ca. 80 r.p.m. to ensure uniform exposure of all parts of the solution, and cooled with an electric fan. Under these conditions the temperature rose immediately to 28°, then increased gradually to 33° during the first hour, and to 35° during the next three. The solutions were irradiated for 5, 10, 15, 30 and 55 min. respectively; 30 min. irradiation caused a slight opalescence, and 55 min. a definite cloudiness of too small a particle size to be retained by fine filter paper. Some of this solution was also irradiated under similar conditions in a pyrex tube of the same dimensions; when a slight opalescence appeared after 4½ hr. the irradiation was stopped.

*Series B.* The same arrangements as in Series A were used, but to minimize the heating of the solution by the arc, and to reduce fluctuations in intensity of irradiation due to erratic cooling of the arc by the electric fan, the lamp was protected by a quartz window. The distance from the arc to the quartz tube was 30 cm. instead of 20 cm. as in Series A. A Photronic cell was placed beneath the rotating tube to detect changes in the amount of radiation falling on the tube.

The solutions in Series B were irradiated for 15 and 30 min., and 1, 2 and 3 hr. The average temperature varied between 27.5 and 31° from experiment to experiment although the maximum deviation during any one irradiation was 2°. The average value of the current given by the Photronic cell was constant from experiment to experiment to within  $\pm 2\%$ . Because of the greater distance of the arc from the solution, 2 hr. irradiation in this series corresponded very nearly to 55 min. irradiation in Series A, but because of the higher pH, the solutions remained clear even after 3 hr. irradiation.

A portion of the solution used in this series was exposed for 3 hr. to the continuous radiation from a soft X-ray tube (Cu target, 100,000 V.), the total dose used being 29,000 r.u. (measured with a Victoreen R-meter). In the absence of a detectable change in the albumin solution, no further irradiations with X-rays were made (see Table I).

*Series C.* To separate the effect of irradiation as far as possible from the effect of heat, the solutions in this series were kept below 1° during irradiation by ice-

water flowing over the rotating quartz tube. The irradiated solutions were stored in ice-water (not frozen, to prevent possible aggregation of the denatured protein) until placed in the centrifuge cell, and ultracentrifuged at the lowest convenient temperature ( $21^{\circ}$ ), in order to minimize possible aggregation during the run. (Clark [1935] found that coagulation of isoelectric egg albumin irradiated at  $4^{\circ}$  has a comparatively high temperature coefficient, but occurs only slowly below  $20^{\circ}$ .) During the 2 hr. irradiation the Photronic cell readings were constant at 70 to within  $\pm 2\%$ ; during the 1 hr. irradiation the average reading dropped to 62, constant to within  $\pm 3\%$ . The lamp burned out before a third irradiation could be made. The radiation doses in this series are thus not proportional to exposure time; allowance has been made for this variation in the graphical presentation of the data (Fig. 2), on the assumption that the intensities of all wave-lengths were reduced in the same ratio. The uncertainty in this assumption does not seriously affect the conclusions drawn.

*Light absorption.* Absorption spectra were obtained using the Hilger echelon cells [Twyman *et al.* 1931-32; Twyman, 1933] with a medium-sized Bausch & Lomb quartz spectrograph, by technique previously described [McDonald, 1936]. The absorption spectra are shown in Fig. 1. The position of the absorption bands are correctly represented but, because of a systematic error in the apparatus [vide Magill *et al.* 1937, Fig. 12], the ordinates do not show the absolute extinction coefficients, but give values which are too high by a factor which varies somewhat from wave-length to wave-length. At wave-lengths corresponding to the absorption maxima the measured values were found to be too large by a factor of 1.1. The corrected heights of the absorption maxima are plotted against the amount of irradiation in Fig. 2 (I, II and III). Using the above correction factor, a value of 6.0 was obtained for the specific extinction coefficient of serum albumin. Svedberg & Sjögren [1930] found a value of *ca.* 5 over the pH range 5-8, and Smith [1929] a value of 5.8 at pH 7.

*Sedimentation velocity.* Measurements of sedimentation velocity were made in a standard Svedberg oil-turbine ultracentrifuge at a speed of 56,000-58,000 r.p.m., corresponding to a centrifugal force of about 215,000 times gravity at the middle of the cell. The temperature of centrifuging was  $24-26^{\circ}$  for Series A and B, and  $21^{\circ}$  for Series C. Sedimentation was followed by the refractive index method of Lamm [1928: 1929] using a cell 3 mm. in thickness and a scale distance of 10 cm. Photographs were taken every 10 min., and the runs were continued for approximately 2 hr. Solvent runs were made from time to time under identical centrifuging conditions [cf. Pedersen, 1936] to provide standards from which the displacements caused by sedimentation of the protein were measured. These displacements were plotted against the distance from the centre of rotation to give the usual sedimentation diagrams.

All experimental conditions were kept as constant as possible from run to run, so that in any series the relative heights of sedimentation curves representing equal times of centrifuging and of diffusion give, to a first approximation, the relative concentrations of albumin of unchanged sedimentation constant in the irradiated solutions. The principal factor reducing the exactness of this relationship is the presence of aggregates incompletely centrifuged from the plane of the boundary, which increase the displacement. This has been minimized by selecting for the comparison a time of centrifuging (50 min.) sufficiently far advanced to ensure fair separation of the heavy particles, but early enough to avoid complications due to reflexion of particles from the bottom of the cell. The relative heights, using 100% for the untreated solution, are given in Fig. 2 (IV and V).

The sedimentation constants were calculated by the usual equation [Svedberg, 1933; 1937]:

$$s_{20} = dx/dt \cdot 1/\omega^2 x_m \cdot \eta_T/\eta_{20} \cdot (1 - V\rho_{20})/(1 - V\rho_T)$$

and are listed in Table I. The sedimentation constant,  $s = 4.5 - 4.6$ , for the untreated material in Series A and for all but two determinations of Series C, is in good agreement with the published value,  $s = 4.5$  [Svedberg, 1937]. For Series B, and two determinations in Series C, the values are lower. This is discussed later.

Representative sedimentation diagrams are shown in Figs. 3 and 4. To avoid repetition, the diagrams for untreated albumin in Series B and C, and for X-ray-treated albumin in Series B, have been omitted; they were similar in appearance and symmetry to that of the untreated solution in Series A.

To estimate the relative amounts of centrifugible material in the various solutions [vide McFarlane, 1935, 1] the areas enclosed by the sedimentation curves were converted into absolute units, and, to correct for the dilution caused by centrifuging in a sector-shaped cell in a field of varying intensity [Svedberg & Rinde, 1924], multiplied by the factor  $(x_i/x_o)^2$ . Since the refractive indices (measured, at 26°, with an Abbé refractometer using a sodium lamp as light source) of the irradiated serum albumin solutions were identical with those of the unirradiated solutions, the corrected areas so obtained are proportional to the concentrations of centrifugible protein. The initial and final areas for each experiment are listed in Table I.

Spreading coefficients were calculated [Lamm, 1929] from the half-width,  $u$ , of the sedimentation curves at a height,  $h$ , where

$$h = \text{maximum height}/\sqrt{e} = 0.606 \text{ maximum height.}$$

The values of  $u^2$  were plotted against the time of centrifuging; the spreading coefficients, or "apparent diffusion constants", were given by the slopes of these curves [Loughborough & Stamm, 1936]. Constant values were obtained for the untreated solutions and for those which showed no marked change; these are listed in Table I. The others showed a drift with time, and single values can therefore not be given. In the solutions irradiated for the longest periods of time (A7, A8, B6, B7, B8, C3) there were discontinuities in the  $u^2$ -time curves; these represent the separation of discrete boundaries composed of particles whose concentration is sufficient to cause pronounced widening of the curve at the height considered.

The spreading coefficients calculated from the curve areas [McFarlane, 1935, 1] are listed in Table I. For characterizing mixtures this method of calculation is preferable to the one given above because it includes the spreading due to all particles present, and not only to those of sufficient concentration to widen the curve appreciably at the height considered. Further, for the untreated solutions, the method gave values which are in better agreement with the published values of the diffusion constant, 6.17 [Svedberg, 1937]. For a heterogeneous mixture the spreading coefficient has no physical meaning, but does provide an index of the range and relative concentrations of particle sizes.

## DISCUSSION

*Absorption spectra.* The absorption spectra (Fig. 1) show that at both pH the light absorption increases regularly as irradiation proceeds; the increase is general for wave-lengths greater than 2400 Å. but most pronounced at the characteristic protein maximum. For the same radiation dose (Fig. 2), the



increase in light absorption is greater at  $pH$  5.4 than at  $pH$  7.4. This suggests that the reactions causing the increase are inhibited at the higher  $pH$ .

As a rule the irradiation of amino-acids, dipeptides and related compounds containing only aliphatic radicals causes increased absorption of wave-lengths less than 2500 Å., while irradiation of those containing aromatic nuclei causes increased absorption at wave-lengths greater than 2400 Å. [Allen *et al.* 1937].

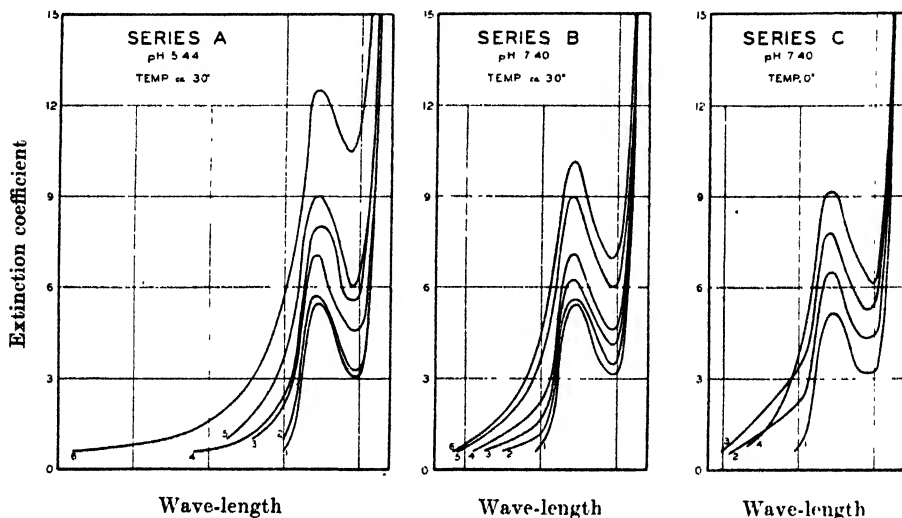


Fig. 1. Increase in absorption spectra with irradiation. Irradiation: Series A, 1, untreated; 2, 10 min.; 3, 15 min.; 4, 4-25 hr. in glass; 5, 30 min.; 6, 55 min. Series B, 1, untreated; 2, 15 min.; 3, 30 min.; 4, 1 hr.; 5, 2 hr.; 6, 3 hr. Series C, 1, untreated; 2, 1 hr.; 3, 2 hr.; 4, 2 months after 2 hr. irradiation.

Too little is known about the absorption spectra of individual amino-acids and polypeptides, and of their photolytic products, to ascertain the specific reactions responsible for the increased absorption of irradiated proteins. In general, u.v. light and cathode rays convert primary and secondary amino-acids into aldehydes, and tertiary amino-acids into ketones, by the addition of oxygen and water and the elimination of ammonia, carbon dioxide and water [Allen *et al.* 1937; Neuberg, 1908; 1910; Harris, 1926; Rao & Dhar, 1934; Becker & Szendrő, 1931]. The rate of ammonia cleavage varies from compound to compound, and is more rapid in an acid than in an alkaline medium [Lieben & Urban, 1931]. Peptones and proteins also yield aldehydes, ammonia and carbon dioxide, a fact which can be explained only by assuming that at some stage of the process hydrolysis of the peptide linkage takes place. Mitchell [1936], from a study of the effect of u.v. light on monolayers of egg albumin, has suggested that the primary reaction is the hydrolysis of CONH linkages adjacent to aromatic residues, with liberation of tyrosine, tryptophan, phenylalanine and perhaps histidine, since these groups are responsible for the high light absorption of the protein in the range 2500-2900 Å.

The concentrations of centrifugible material in the irradiated solutions also point to a reaction of this character. Table I gives the areas enclosed by the sedimentation curves, to which the concentrations of centrifugible material are proportional. All solutions which remained clear, and from which, therefore, none of the albumin had been precipitated, gave *initial* areas that were approxi-

mately the same as for unirradiated albumin. This shows that the non-centrifugible material split off by the action of the light constituted only a few % of the total weight. Tyrosine and tryptophan comprise respectively 4.66 and 0.53 % of the weight of serum albumin [Folin & Marenzi, 1929], phenylalanine 2.3 % [Stenström & Rheinhard, 1925], histidine 1.95 % [Block, 1934]; together they amount to about 9.4 %. The sedimentation data (Fig. 2, V) show that 3 hr. irradiation at pH 7.4 caused modification (with respect to sedimentation constant) of about 70 % of the serum albumin. Complete hydrolysis of all aromatic amino-acids in this modified fraction of the albumin would represent, therefore, a loss of about 6.6 % as non-centrifugible material. The observed loss, calculated from decrease in initial area (Table I), is not more than 4.5 % and is probably less, since part of the drop in initial area may be due to the removal of a small concentration of heavy aggregates. The diminution in area is thus consistent with the view that only aromatic amino-acids are hydrolysed from the molecule.

However, this reaction cannot be responsible for the increased light absorption. Tyrosine, tryptophan and phenylalanine have molecular extinction coefficients of 1576, 5900 and 180 respectively [Gróh & Hánák, 1930]. Calculation, using the above figures, shows that a solution containing as free acids the same concentrations of these amino-acids as are present in a 1 % solution of serum albumin would have an extinction coefficient of 5.83, while the specific extinction coefficient of serum albumin is between 5.0 and 6.0 [Svedberg & Sjögren, 1930; Smith, 1929].

Arnow [1935] has shown that irradiation of egg albumin with  $\alpha$ -particles causes increased absorption in isoelectric or acid solutions, decreased absorption in solutions alkaline to the isoelectric point. He has suggested that the increase may result from the photo-oxidation of phenylalanine to tyrosine and of tyrosine to dihydroxyphenylalanine ("dopa") which has a molecular extinction coefficient of 2500 [Abderhalden & Rossner, 1928]. Recently he has demonstrated that low concentrations of "dopa" are formed by the action of u.v. light on tyrosine [Arnow, 1937]. However, the conversion into "dopa" of all the tyrosine and phenylalanine present in serum albumin would cause only a 95 % increase in the extinction coefficient. This mechanism, therefore, even with the improbable assumption of complete conversion, is inadequate, by itself, to account for the increases in light absorption observed in the present investigation.

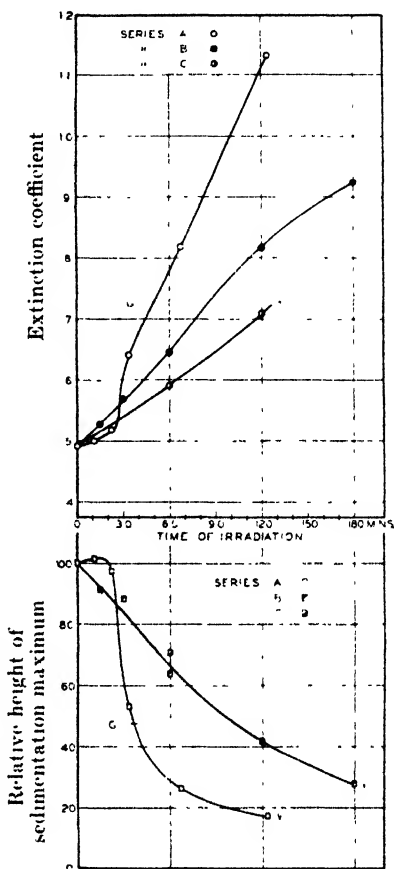


Fig. 2. Correlation between extinction coefficient and relative height of sedimentation maximum. The abscissae represent actual times of irradiation for Series B and C. The times for Series A are the actual irradiation times  $\times 9.4$ , to compensate for the change in distance of the light source.  $G_1$  and  $G_2$  give the extinction coefficient and relative sedimentation height, respectively, of the solution irradiated 4.25 hr. in glass.

Table I

Treatment	Appearance	$\epsilon_{20} \times 10^{13}$	Spreading coefficient		Curve areas	
			Area	Width	Initial	Final
					sq. cm.	
Series A. $pH = 5.44$ ; temp. approx. $30^\circ$						
1. Untreated (before)	Clear	4.67	6.53	5.4	0.0047*	
2. Untreated (after)	Clear	4.50	6.15	5.3	0.0047*	
3. Glass 4-25 hr.	Opalescent	4.84	12.8	—	0.0034	0.0034
4. Quartz 5 min.	Clear	4.49	5.59	5.4	0.0047	0.0047
5. Quartz 10 min.	Clear	4.61	7.19	5.8	0.0047	0.0047
6. Quartz 15 min.	Clear	4.89	16.8	—	0.0046	0.0037
7. Quartz 30 min.	Opalescent	4.98	24.8	—	0.0026	0.0020
8. Quartz 55 min.	Cloudy	4.92	18.7	—	0.0016	0.0013
Series B. $pH = 7.40$ ; temp. approx. $30^\circ$						
1. Untreated (before)	Clear	4.41	5.33	4.0	0.0046*	
2. Untreated (after)	Clear	4.47	5.25	4.0	0.0046*	
3. Quartz 15 min.	Clear	4.41	7.08	—	0.0046	0.0044†
4. Quartz 30 min.	Clear	4.29	7.44	—	0.0046	0.0043†
5. Quartz 1 hr.	Clear	4.44	16.9	—	0.0046	0.0039†
6. Quartz 2 hr.	Clear	4.41	48.5	—	0.0046	0.0036‡
7. Quartz 3 hr.	Clear	4.41	73.0	—	0.0044	0.0031§
8. Quartz 3 hr.	Clear	4.33	80.0	—	0.0044	0.0035§
(repeated 6 days later)						
9. X-rays, 29,000 r.u.	Clear	4.39	6.24	3.7	0.0046*	
Series C. $pH = 7.40$ ; temp. $0^\circ$						
1. Untreated (before)	Clear	4.63	6.61	3.5	0.0048	
2. Quartz 1 hr.	Clear	4.57	17.1	—	0.0046	0.0040†
3. Quartz 2 hr.	Clear	4.57	54.6	—	0.0046	0.0035‡
4. Heated, and rotated for 3 hr.	Clear	4.22	4.69	—	0.0071*	
5. Untreated (temp. of centrifuging, $25-26^\circ$ )	Clear	4.23	4.72	—	0.0075*	
6. Untreated (temp. of centrifuging, $20-22^\circ$ )	Clear	4.53	6.02	—	0.0076*	

\* Average values over entire time of centrifuging: † 72 min., ‡ 60 min. and § 50 min. from start of centrifuging.  
 || Albumin concentration greater than 0.81 %.

Immediately after irradiation, the solutions irradiated at  $0^\circ$  (Series C) showed less increase in absorption at the protein maximum, but more increase on the long-wave side of the protein band, than the solutions of the same  $pH$  irradiated for the same periods of time at  $30^\circ$  (Series B) (compare B4 and C2, B5 and C3, Fig. 1). Prolonged standing at  $3^\circ$  led to the disappearance of this difference, resulting in increased absorption below 3000 Å. and decreased absorption above 3000 Å. in both series of solutions, but to a far more limited extent in Series B than in Series C (compare B5, C3 and C4, Fig. 1). The effect of the temperature of irradiation was in this way eliminated, and identical spectra were obtained for the two series, a finding which is consistent with the observations of Rajewsky [1929; 1930] and Clark [1935] that the temperature coefficient of the primary photochemical process is unity. Since no changes could be detected in the absorption spectra of the unirradiated solutions which had been stored at  $3^\circ$  for the same lengths of time, it seems possible that part of the absorption at wave-lengths longer than 3000 Å., observed in the solutions irradiated at  $0^\circ$ , represents an unstable intermediate stage [Mirsky & Pauling, 1936, p. 444] which produces, on warming or on prolonged standing in the cold, the compound(s) responsible for the increased absorption at the protein maximum.

The two solutions irradiated 2 hr. at  $pH$  7.4 were brought to  $pH$  5.4 by dialysis at  $3^\circ$  through cellophane membranes against large volumes of  $M/15$  phos-

phate buffer. The absorption spectra were taken first with the chilled solution, then again after warming to 30° for 20 min. Both solutions remained clear. Each gave the same spectrum before and after warming, but the two differed a little from each other, perhaps because of slight dilution during dialysis. The increased absorption, above that of the native protein, was in both instances reduced to about half what it had been before dialysis, i.e. to an extinction coefficient of 7. This suggests that while part of the increase results from cleavage products small enough to diffuse through cellophane membranes, much of it must arise either from reactions in the protein molecule without cleavage, or from cleavage products too large to diffuse through cellophane.

The solution of pH 7.4 irradiated 1 hr. at 30° was brought to pH 5.4 by the addition at 0° of  $N HCl$ ; this caused a 4% increase in volume. Within the limits of error of the method, the absorption spectra measured before and after warming were identical with each other and with that of the solution before dialysis. Since the solution which was given approximately the same radiation dosage at pH 5.4 (Series A, 30 min.) gave much greater absorption, it appears that the action of the light is inhibited at pH 7.4.

*Sedimentation velocity measurements.* The most obvious result of irradiation is the progressive reduction in the heights of the sedimentation curves (Figs. 3 and 4): but in each series the sedimentation constants remain the same as for the native protein (Table I), except for a slight increase at pH 5.4 (Series A). These facts show that in the irradiated solutions the boundaries represent albumin of unchanged sedimentation constant, and that the concentration of such albumin is diminished by successively longer periods of irradiation. Irradiation also causes an asymmetrical broadening of the sedimentation curves to the right of the maximum, which demonstrates the presence of particles heavier than native albumin, and suggests that the protein molecules which have been attacked by the light coalesce to form aggregates. Resolution of the faster-moving boundaries was not sufficient for calculation of the sedimentation constants, but qualitative information concerning aggregate sizes can be gained from the curve areas and curve spreading.

For solutions irradiated longest, the curve areas (Table I) diminish as centrifuging proceeds, because the heavy aggregates, in order of size, have been centrifuged to the bottom of the cell and removed from the field of observation. At pH 7.4 the rate of this reduction increases with increase in irradiation time; this shows that as irradiation proceeds not only the concentrations but also the average sizes of the aggregates become greater. At pH 5.4 the separation of a visible precipitate causes a reduction in *initial* area, the greater the longer the effective period of irradiation; the further reduction in area during centrifuging shows that the heavy aggregates characteristic of the solutions irradiated 15 and 30 min. are present in lower absolute concentration in the solution irradiated 55 min., and are almost entirely absent from the solution irradiated in pyrex glass for 4½ hr. The spreading coefficients show the same trend, a continuous increase at pH 7.4, and at pH 5.4 an increase to a maximum near 30 min. irradiation: but the greater magnitude of the variations emphasizes the differences in average particle size. These differences were also clearly shown by the variation of  $u^2$ , the square of the half-width of the curve at 0.606 times the maximum height, with time of centrifuging: at pH 7.4 each solution irradiated for 30 min. or more gave curves which indicated progressively diminished spreading, the result of the removal of the heaviest aggregates as the centrifuging proceeded, while at pH 5.4 the solutions irradiated for 30 and 55 min. both gave curves which indicated the gradual separation of particles sedimenting with velocities of the

same order of magnitude, and the absence of appreciable concentrations of heavy particles. The discontinuities in the  $u^2$ -time curves for the solutions irradiated longest (A7, A8, B6, B7, B8, C3) represent the separation of discrete boundaries each composed of particles whose concentration is sufficient to cause marked

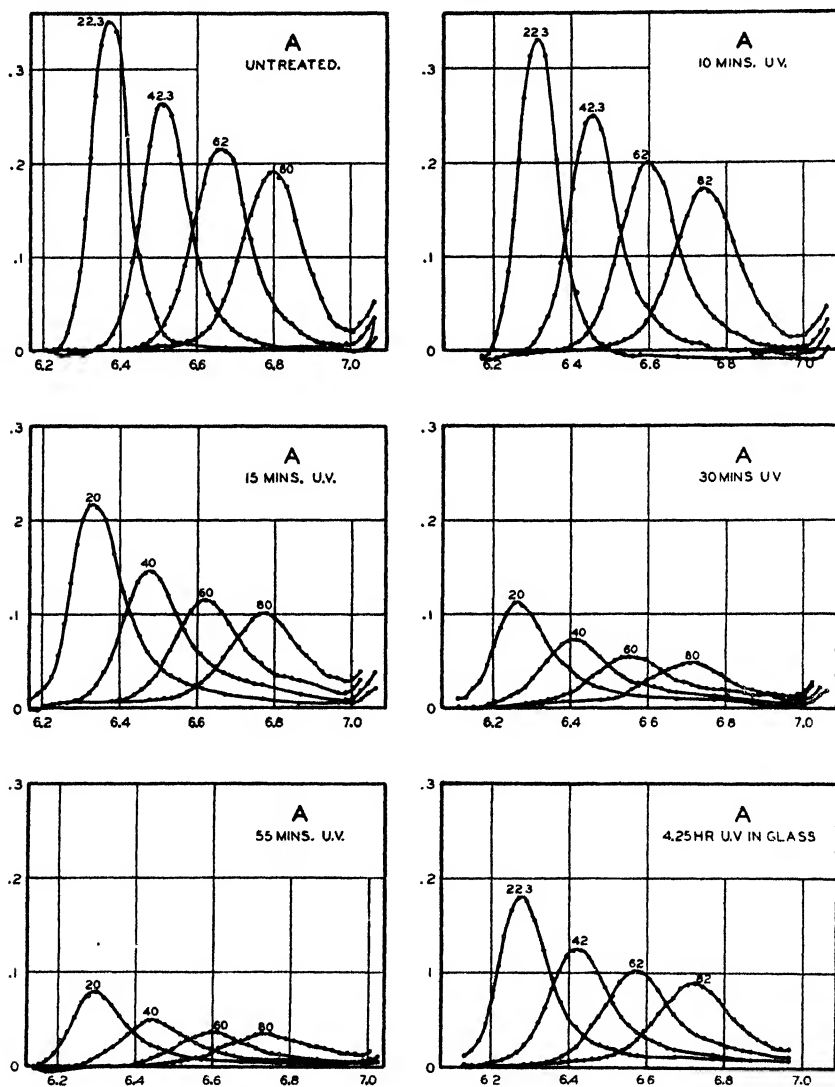


Fig. 3. Sedimentation diagrams. Series A. pH 5.44. Abscissae, distance from centre of rotation ( $X$  in cm.); ordinates, scale-line displacement ( $Z$  in mm.). The figures near the curve maxima represent the time in minutes from the application of the final driving pressure to the rotor. The figure under the series letter shows the duration of u.v. irradiation. Temperature of irradiation *ca.* 30°.

broadening of the curve at the height considered. The approximate sedimentation constants for these boundaries, estimated from the discontinuities, fell into two groups, one about 6, the other a little more than 7, and probably correspond to aggregates composed of two or three albumin residues.

The results as a whole indicate that in solutions sufficiently alkaline to prevent precipitation of the denatured protein, irradiation leads to the formation of increasing amounts of aggregated product of successively greater particle size,

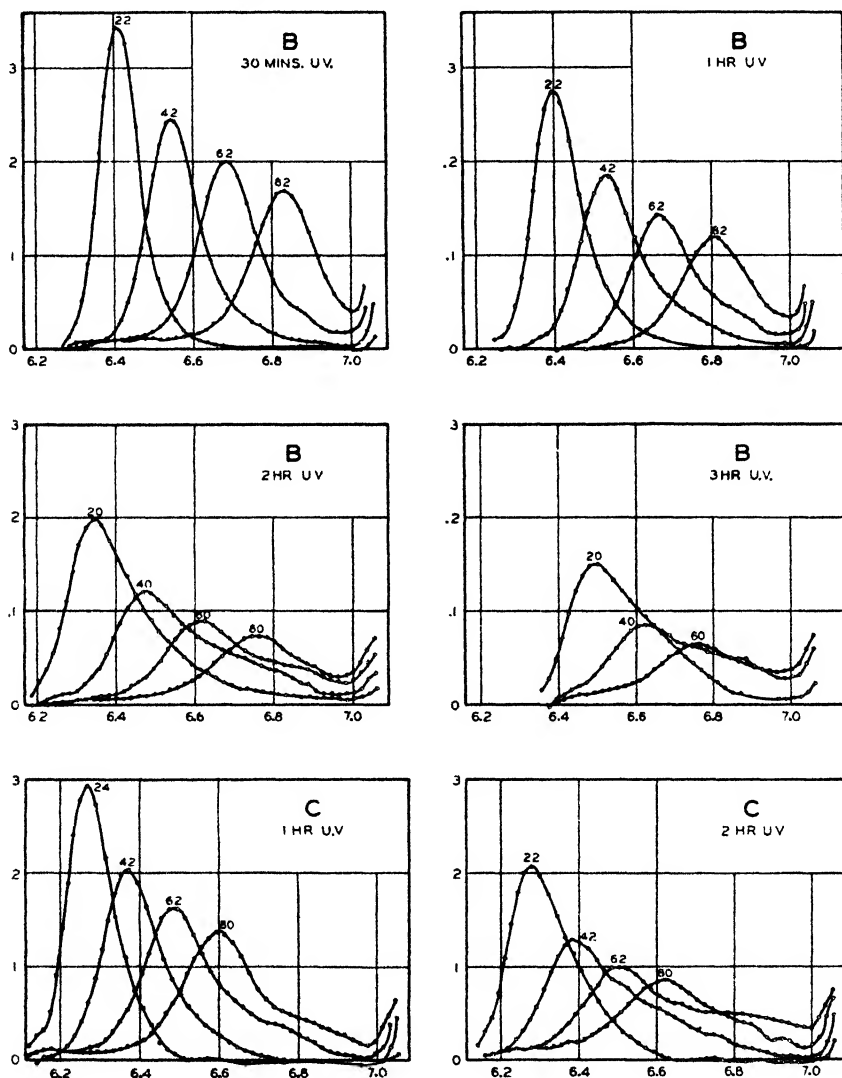


Fig. 4. Sedimentation diagrams. Series B and C. pH 7.40. Abscissae, distance from centre of rotation ( $X$  in cm.); ordinates, scale-line displacement ( $Z$  in mm.). The figures near the curve maxima represent the time in minutes from the application of the final driving pressure to the rotor. The figure under the series letter shows the duration of u.v. irradiation. Temperature of irradiation: Series B, ca.  $30^\circ$ ; Series C,  $0^\circ$ .

but there appears to be a limit to the size developed. This type of aggregation seems to take place even at low temperatures, or else very rapidly at about  $20^\circ$ , because the precaution, observed in Series C, of irradiating and storing at  $0^\circ$  until the beginning of the ultracentrifuge run, had negligible effect on the aggre-

gation; this can be seen from the similarity of the sedimentation curves (Fig. 4), of the relative heights of the sedimentation maxima (Fig. 2, V) and of the spreading coefficients (Table I), to those of the solutions irradiated for the same times at 30°.

On the other hand, in solutions nearer to the isoelectric point there is no limit to the particle size, and a visible precipitate is formed at the expense of aggregates of intermediate size. Here the comparatively high temperature of irradiation (30°) has definitely influenced the particle size distribution. Because the coagulation process has a rather large temperature coefficient [Rajewsky, 1929; 1930; Clark, 1935], the solutions irradiated the longest were given the most favourable conditions for coagulation, and became most impoverished with respect to soluble aggregates, especially those of large size. The maximum in the spreading coefficient after about 30 min. irradiation in quartz, therefore, probably represents the stage of aggregation which immediately precedes the formation of a visible precipitate, first seen after 30 min. irradiation.

The absence of heavy soluble aggregates from the solution irradiated for 4½ hr. in pyrex glass is undoubtedly the result of prolonged high temperature. The absorption spectrum of this solution is in all respects similar to those of the solutions irradiated in quartz. Since, as can be seen from Fig. 2 (I and IV), the heights of the sedimentation and absorption maxima correspond to about the same degree of change as that to be expected from approximately 20 min. irradiation in quartz, it seems reasonable to suppose that the reaction is the same in both quartz and pyrex, but that the difference in extent results from the greater transparency of quartz to the effective wave-lengths [Rajewsky, 1930].

The greater uniformity of the sedimentation constants for the observed boundary in solutions irradiated at pH 7.4 can probably be attributed to the preponderance among the aggregates of heavy particles which quickly separate from the main boundary; the upward trend at pH 5.4, to the higher relative concentrations of small aggregates, which, being incompletely resolved from the main boundary, cause an apparent increase in the sedimentation constant.

For equal irradiation at 30°, the reduction in the concentration of albumin of unchanged sedimentation constant is greater at pH 5.4 than at pH 7.4; this is shown in Fig. 2 (IV and V). It appears, therefore, that the aggregation process is inhibited at the higher pH. Gross coagulation, which is also inhibited at pH removed from the isoelectric point, is known to be a process distinct from the light reaction [Rajewsky, 1929; 1930; Clark, 1935]. Doubtless, therefore, even the simplest stages of aggregation must also be considered separate from it, though it is scarcely justifiable to conclude on this basis alone that the initial photochemical process, which may be considered to render the molecules susceptible to aggregation, is also inhibited at the higher pH. However, the correlation between reduction in albumin concentration and increase of light absorption, shown in Fig. 2, is fairly convincing evidence that the two are manifestations of the same fundamental change. It has already been shown that the reaction causing increase of light absorption is inhibited at pH 7.4: it thus seems likely that the photochemical reaction causing aggregation may also be repressed. This conclusion is supported by the fact that while solutions irradiated at pH 5.4 for 30 and 55 min. respectively showed opalescence and turbidity, the solutions which were adjusted to pH 5.4 after receiving equivalent amounts of radiation (1 and 2 hr.) at pH 7.4 all remained clear.

Young [1922], from measurements of the amounts of coagulum formed at the isoelectric point after irradiation at various pH, reached the opposite conclusion, that the reaction was progressively more rapid on both sides of the isoelectric

point. However, his solutions were not rotated during irradiation, and since the increase of light absorption has been shown to be less at  $pH$  7.4 than at  $pH$  5.4, a possible reason for his results may be the lower screening effect, at  $pH$  removed from the isoelectric point, of the irradiated layers nearest the light source.

Because of the uncertainty of the protein concentrations and radiation doses in Series C, a detailed quantitative comparison cannot be made with the results of Series A and B. However, the relative heights of the sedimentation maxima are, as far as can be ascertained, the same as for the solutions of the same  $pH$  irradiated at 30° (Fig. 2, V), while the maximum extinction coefficients are definitely lower (Fig. 2, III). The correlation between rise of absorption maximum and drop in sedimentation maximum is thus not apparent for Series C, the reason probably being that the absorption spectra, taken within 5 min. of the removal of the solution from the ice-bath, represent quite accurately the unheated solutions, while the ultracentrifuge data refer to the solutions warmed to 20–22° for about an hour during the loading and accelerating of the ultracentrifuge. After prolonged standing, during which the temperature had at one time risen to approximately 20° for about an hour, the solution irradiated 2 hr. gave an absorption spectrum identical with that of the solution irradiated 2 hr. at 30°. It appears that solutions of  $pH$  7.4 irradiated at 0° tend very readily to assume the same state, with respect to both aggregation and light absorption, as those irradiated at 30°. It would thus seem, too, that the preliminary heating to 56° of the albumin solutions in Series A and B did not affect the reactions caused by the u.v. light.

Pedersen [1931] found that an irradiated solution of serum albumin of  $pH$  3.5 gave a sedimentation constant about twice that of native albumin, presumably about 9. The apparent discrepancy with the present results can most probably be explained either by the difference in  $pH$  or by the greater boundary-resolving power of the refractive index method as compared with the light absorption method used by Pedersen, especially in the presence of non-centrifugible material of high light absorption. It is possible that in his experiments the unaltered albumin did not appear as a separate boundary, but was completely merged with the aggregation products, so that the measured sedimentation constant represented an average for the mixture as a whole.

The characteristic odour which has been mentioned by several investigators [Pedersen, 1931; Stedman & Mendel, 1926] was absent from our solutions. We are inclined to attribute this to the precaution of rotating the irradiation tube, which ensured more equal exposure of all parts of the solution, and prevented the accumulation of degradation products of high light absorption in the layers nearest to the source of illumination.

To determine whether rotation of the albumin solution caused any surface denaturation contributing to the observed results, especially in the solutions which had been heated to 56°, and which might, therefore, be more susceptible to denaturation, some of the solution used for Series C was heated to 56° for 25 min., rotated without irradiation for 3 hr. at 26–29° and ultracentrifuged at 25°. The sedimentation curves were similar in appearance and symmetry to those given by the untreated albumin, and showed no evidence of the aggregation which might be expected to result from denaturation. There was a drop in the sedimentation constant, beyond the limits of experimental error, from 4.6 to 4.2, and a drop in spreading coefficient,  $D$ , from 6.6 to 4.7 (see Table I). but further experiments showed that heating was the cause, not rotation. A sample of the untreated solution ultracentrifuged at a cell temperature of 25–26° gave the same



low constants,  $s=4.2$ ,  $D=4.7$ ; while a further sample centrifuged at  $20-22^\circ$  gave higher values,  $s=4.5$ ,  $D=6.0$ , approximately those originally observed. The conclusion was drawn, therefore, that in the irradiation experiments the results were not affected by the rotation, and were due solely to the u.v. light.

The sedimentation constant of human serum albumin appears to be definitely lower at  $pH\ 7.4$  than in the region of the isoelectric point, as can be seen by comparison of Series B with the unirradiated solutions of Series A (Table I). In the solution used for Series C, the change to  $pH\ 7.4$  from the isoelectric point was made at a temperature which certainly did not exceed  $15^\circ$ ; the solution was subsequently kept frozen and thawed only for removal of samples. In all the solutions of this series, whether irradiated or not, the temperature of which had never risen above  $22^\circ$ , the sedimentation constant was that of isoelectric serum albumin,  $s=4.5-4.6$ , also observed in Series A, instead of the lower value which would be expected from the increase in  $pH$ ; for the unirradiated solutions of this group, the spreading coefficients, too, were the same as in Series A,  $D=6.0-6.6$ . The lower values appeared only in the two samples which had been heated to  $56^\circ$  and  $25-26^\circ$  respectively. It is believed, therefore, that whatever the nature of the change producing these differences, it must occur at a negligible rate below  $22-23^\circ$ , but rapidly at  $25^\circ$  and above. In view of the very high temperature coefficients of many protein reactions, such a hypothesis is not theoretically unreasonable.

At  $pH\ 9.5$  the molecular weight of lactoglobulin is the same as at the isoelectric point, but both the sedimentation constant and diffusion constant are lower by about 6% [Lamm & Polson, 1936]; this difference has been attributed to a change in the shape of the molecule, resulting in a higher frictional constant. Human serum albumin probably also acquires a higher frictional constant without change in molecular weight when brought to  $pH\ 7.4$  from the isoelectric point, either because of a change in shape or because of a change in the degree of hydration.

While the values for the sedimentation constant and spreading coefficient for Series A are in good agreement with those of the unheated solutions in Series C, those of Series B, instead of agreeing with those of the heated solutions in Series C, lie intermediate between them and the isoelectric values. A possible reason for this discrepancy may be the greater age of the stock solution used for Series B, but the more probable explanation is the greater concentration of the heated solutions in Series C. The diffusion constant of serum albumin near the isoelectric point is substantially independent of concentration up to 2% [Lamm & Polson, 1936]; but it is possible that at  $pH\ 7.4$ , where the frictional constant is higher, increased concentration may more definitely repress both sedimentation and diffusion.

#### SUMMARY

1. The effect of u.v. irradiation on 0.8% solutions of purified human serum albumin at  $pH\ 5.4$  (1% NaCl) at about  $30^\circ$ , and at  $pH\ 7.4$  ( $M/15$  phosphate buffer) at  $30$  and  $0^\circ$ , was studied by means of sedimentation velocity and light absorption measurements.

2. Irradiation caused increased light absorption at both  $pH$ , general for wave-lengths longer than  $2400\text{ \AA}$ ., but greatest at the protein band; the increase was greater at  $pH\ 5.4$  than at  $pH\ 7.4$ . At  $pH\ 7.4$ , irradiation at  $0^\circ$  caused more increase for wave-lengths above  $3000\text{ \AA}$ ., but less increase for wave-lengths below  $3000\text{ \AA}$ ., than at  $30^\circ$ ; after prolonged standing in the cold, the two gave identical spectra.

3. The results indicate that irradiation causes the liberation of substances of small molecular weight, and that it also leads to ill-defined photo-oxidation reactions which result in increased light absorption. It is possible that these reactions may involve both the albumin residues and the low-molecular cleavage products.

4. Though visible coagulation occurred only at  $pH$  5.4, irradiation caused aggregation of the protein at both  $pH$ , and gradual reduction in the concentration of unchanged albumin. Aggregation was less pronounced at  $pH$  7.4 than at  $pH$  5.4, and a larger concentration of light-denatured molecules remained unchanged with respect to sedimentation constant. In the absence of a visible precipitate, irradiation led to the formation of successively heavier soluble aggregates, while when visible coagulation occurred the solution was impoverished with respect to soluble aggregates of large size.

5. A correlation was observed between increase of light absorption and decrease in concentration of unaggregated albumin in the irradiated solutions.

6. The sedimentation constant of the unaggregated protein in the irradiated solutions remained essentially that of native albumin.

7. Three hours' irradiation with soft X-rays (29,000 r.u.) caused no detectable change in the sedimentation behaviour or the absorption spectrum.

8. The sedimentation constant of serum albumin is lower at  $pH$  7.4 than near the isoelectric point: the change (probably in the shape or degree of hydration of the molecule) which causes this difference, appears to be prevented at low temperatures and to occur rapidly only at temperatures above  $23^{\circ}$ .

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## II. A STUDY OF THE PASSAGE OF FATTY ACIDS OF FOOD INTO LIPINS AND GLYCERIDES OF THE BODY USING DEUTERIUM AS AN INDICATOR

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It was shown by Schoenheimer & Rittenberg [1935] that fat containing deuterium when fed to mice may subsequently be found in both the "organ fat" and the "body fat" of the animals. The present authors [1936], after administering an oil containing D to a rat for 7 days, also showed that the lipins of the liver and kidney contained fatty acids in which D was present. This suggests that the lipins may play an active part in fat metabolism and not merely a passive role as constituents of protoplasm which are important because of their physical properties.

Sinclair [1935] has come to a similar conclusion as a result of experiments in which the administration of a fat containing elaidic acid led to the appearance of this acid in the lipins of liver and muscle. In Sinclair's experiments one day was the shortest period that was allowed to elapse after the fat feeding before the organs of the animals were taken for examination. It appeared to us that a shorter period than this might yield results of interest and, further, the greater ease with which D can be detected as compared with elaidic acid made its use especially advantageous for this type of work.

### EXPERIMENTAL

The "deutero-fat" was obtained by partially "deuterating" pure unboiled linseed oil with 100 % deuterium (electrolytic) using palladium black as catalyst. About 100 g. oil were treated at a time, the gas (in glass bulbs) was initially at atmospheric pressure, and the apparatus was entirely of glass with the exception of one short connexion of flexible metal tubing joined to the glass by piccined cones. The oil was heated electrically to about 70° and shaken mechanically during the reaction, the progress of which was observed manometrically. It was found possible to introduce D to the extent of 4-5 atoms % of the H present without solidifying the fat or making it unpalatable to the rats.

Groups of 3 or 4 rats were used according to the size of the animals. The total weight of the group was usually about 1 kg. They had been kept on the ordinary laboratory diet before the experiment. Each group was starved for a day before receiving the food containing "deutero-fat". This consisted of 4 g. crushed dog biscuit, moistened with 10 ml. water, and in this 1.9 g. of the deutero-fat were incorporated. The food was usually well taken and was eaten within 3 hr. At the appropriate time (6, 10 or 24 hr.) after giving the food the animals were stunned and killed by bleeding. The blood was collected with addition of oxalate and centrifuged to separate corpuscles and plasma. The liver, kidneys and brain were

removed and a sample of adipose tissue from the abdomen was taken. The tissues or organs of the whole group were worked up together. The adipose tissue was heated with about its own wt. of 50 % KOH and 10 ml. alcohol until saponification was complete. The alcohol was then removed on the water bath, the soaps taken up in water and acidified with dilute  $\text{H}_2\text{SO}_4$ . The liberated fatty acids were taken up in light petroleum and a portion of the solution evaporated. The last traces of the solvent were removed by heating to  $100^\circ$  under reduced pressure in an atmosphere of  $\text{H}_2$  for  $\frac{1}{2}$  hr. The liver, kidneys, brain, blood corpuscles and blood plasma were dealt with as follows. The organs after chopping with a razor were ground up in a mortar and extracted in the cold for 24 hr. with 4–5 times their wt. of alcohol—the blood corpuscles and blood plasma were added directly to the alcohol. The extraction was repeated with absolute alcohol for at least another 24 hr. The tissue substance after removal of the 2nd extract by filtration was dried in a vacuum desiccator over  $\text{H}_2\text{SO}_4$  and then extracted with ether for 8 hr. in a Soxhlet apparatus. The two alcohol extracts were evaporated to dryness under reduced pressure in a current of  $\text{H}_2$  and the residue extracted with dry ether. This extract was added to the Soxhlet extract and the whole distilled to remove the ether. With the liver it was found convenient at this stage to continue with only one-fourth of the extract. The residue containing all the ether-soluble substances of the organ (or blood fraction) was dissolved in 4 ml. dry ether and precipitated with 20 ml. acetone. The precipitate was filtered off, washed with acetone-ether and dried in a vacuum desiccator. It was then taken up in dry ether and the solution evaporated in a weighed flask. The acetone-soluble material was recovered in a similar manner by distillation from a tared flask. In these two fractions the D content was estimated as described below. The acetone-insoluble fraction consists essentially of the lipins of the tissue, and the acetone-soluble fraction of the glycerides and sterols.

*Combustion technique.* The commercial  $\text{O}_2$  used in the combustions was known to be liable to contain traces of oil, and for this reason, as well as on general grounds, the dried gas was purified by submitting it to exactly the same treatment as it would subsequently encounter in the combustion tube, i.e. it was passed through a duplicate tube similarly packed and heated and was then dried before entering the combustion tube proper. In the earlier combustions Pregl's "universal filling" was used in supramax glass tubes. The too hygroscopic lead peroxide was omitted since interchange of water from one combustion to another was not permissible, but otherwise, as regards temp., rate of  $\text{O}_2$ -flow etc., the essential conditions of the Pregl technique were observed, though the heating was electrical. Later it was found convenient to use quartz combustion tubes, omitting the lead chromate from the filling and raising the temperature somewhat ( $750$ – $800^\circ$ ). The tubes (Fig. 1) were wound directly with nichrome ribbon and then boxed in with thick alundum cement. The current (A.C.) was controlled by rheostat and ammeter and the temperature inside the lagging observed by means of a thermocouple.

Since, of the products of combustion, only the water was required and this in the free form, the emergent gases were passed through a glass spiral (*H*) immersed in a "slush" of solid carbon dioxide and alcohol. (A similar cooled spiral followed by two U-tubes packed with  $\text{Mg}(\text{ClO}_4)_2$  was used for drying the  $\text{O}_2$  stream between the two combustion tubes.) There were no rubber connexions, a pieceined ground stopper (*G*) permitted the introduction of the Pt boat containing the sample for analysis, and on completion of the combustion, glass taps served to cut off the  $\text{O}_2$  stream (at *A*) and its exit (at *B*) and to connect the combustion tube (at *C*) to the Hg vapour pump. Moderate evacuation then sufficed to enable the water

sample to be rapidly distilled through a large tap (*D*) into a separate compartment of the apparatus where it was condensed in a detachable "water vessel" of the type shown (*E*), and then resublimed from  $-15^{\circ}$  to  $-30^{\circ}$  into a second vessel (*F*) under a better vacuum.

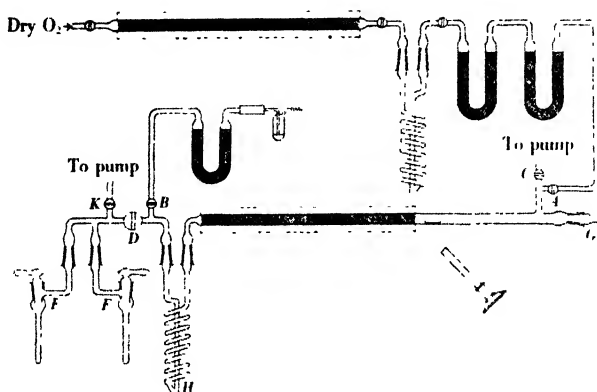


Fig. 1.

*Purification of water from combustion.* About half of the samples dealt with were known to contain N and the rest might contain small amounts, but since this was in the form of substituted  $\text{NH}_3$  it was not at first thought necessary to supplement the filling by Cu for the decomposition of oxides of N. However, a peculiarity in the behaviour of the collected water during sublimation led to tests which revealed the presence of  $\text{HNO}_3$  in appreciable quantities in the collected water. It was therefore decided to use the Cu catalyst, but to do so in a separate apparatus, so as to avoid the necessity of frequent reduction of the Cu. This apparatus was essentially similar to the main combustion apparatus (lower part of Fig. 1) without the provision of the  $\text{O}_2$  stream, with a ground joint for the attachment of a "water vessel" in place of *G*, a narrow U-tube in place of *H*, and the Cu catalyst alone as filling. The latter was maintained (electrically) at a mild red heat and the whole apparatus thoroughly evacuated before each experiment. The water sample from a combustion attached at *G* was first cooled to  $-80^\circ$  while the evacuation was completed, and then (taps *C* and *D* being closed) allowed to warm up in stages to room temp. while the U-tube was cooled. The controlled distillation over the heated catalyst was accelerated in its later stages by pumping off permanent gases through *D* and *K*. The purification of the water sample was then completed by a double vacuum sublimation (between  $-15^\circ$  and  $-30^\circ$ ) to the receivers *E* and *F*. As a check the water samples were always tested for neutrality after the densities had been determined.

**Density determination.** The purified water samples were generally about 10 mg. but in several cases as small as 4–5 mg. Their densities were determined by the method of Gilfillan & Polanyi [1933] to a precision of one part in 100,000, and the atomic % of D calculated to 0.01 %. As a check on the combustion technique samples of ox-liver lecithin free from D were similarly treated, one such control being inserted after every two of the ordinary combustions. Similar controls were used initially in perfecting the technique and afterwards—throughout the recorded experiments—they yielded water samples of normal density within the precision of measurement.

## RESULTS

The % of the "glyceride" and "lipin" fractions obtained from the organs or tissues and the D contents of these fractions are given in Tables I and II respectively. The % figures for both fractions are reckoned on the wt. of the fresh tissue except with the blood corpuscles and plasma, which are based on 100 ml. The D figures represent atoms % D in the crude "glyceride" and "lipin" fractions. The fat administered contained 4.87 atoms % D.

Table I. *Glyceride fractions*

Organ or tissue	Glyceride %			Atoms % D in glycerides		
	6 hr.	10 hr.	24 hr.	6 hr.	10 hr.	24 hr.
Liver	1.26	1.41	1.55	0.86	0.67	0.30
Kidney	1.27	1.43	—	0.12	0.22	0.20
Brain	2.98	2.87	2.50	0.01	0.05	0.025
Blood corpuscles	0.32	0.43	0.50	0.67	0.12	0.17
Blood plasma	0.23	0.28	0.19	1.26	0.64	—
Adipose tissue	—	—	—	0.06	0.03	0.13

Table II. *Lipin fractions*

Organ or tissue	Lipin %			Atoms % D in lipins		
	6 hr.	10 hr.	24 hr.	6 hr.	10 hr.	24 hr.
Liver	3.97	4.42	4.11	0.47	0.52	0.42
Kidney	—	2.85	2.79	0.16	0.14	0.17
Brain	4.0	—	4.39	0.004	0.08	0.03
Blood corpuscles	0.59	0.40	0.26	0.12	0.10	—
Blood plasma	0.07	—	0.08	0.12	0.47	—

## DISCUSSION

Acetone precipitation does not produce a sharp separation of lipins from glycerides so that the experiments give comparative rather than strictly quantitative indications of the distribution of the deuterio-fatty acids between glycerides and lipins after absorption. Perhaps the figures of greatest interest are those obtained 6 hr. after administration of the fat. At this time the fatty substances of all the tissues examined showed the presence of D. As would be expected the highest % D was found in the glycerides of the blood plasma and following these in descending order come those of liver, blood corpuscles and kidney. In the brain and adipose tissue D was only just detectable. The % D in the liver lipins and glycerides was very much greater than in those of the kidney, which accords with the generally accepted views on the special part played by the liver in fat metabolism. A comparison of the % D in the various organ fats with that in the fat administered shows that at the end of 6 hr. about 26 % of the plasma glyceride, 18 % of the liver glyceride and 2.5 % of the kidney glyceride was derived from the deuterio-fat administered. This indicates a selective intake of fat from the blood by the liver soon after its absorption. The lipins of the liver also participate in this phenomenon. Although the % D in the liver lipins is lower than that in of the glycerides, if one takes into account the smaller proportion of fatty acids which they contain (65 %) it is seen that about 14 % of the lipin fatty acids had come from the deuterio-fat of the food.

The figures obtained after 10 hr. show a decline in the D content of the blood fat. This might be expected from previous observations on fat absorption. Owing to technical difficulties the 24 hr. figures for the plasma and corpuscles were not

obtained. In the liver there is a gradual decline in the % D in the glycerides during 24 hr. but much less change in that of the lipins. In the kidney the glyceride figures at 10 and 24 hr. are about the same and higher than the 6 hr. value, whereas the lipin D does not change significantly. The kidney, therefore, does not appear to respond to the changes in blood fat either as rapidly or to the same extent as the liver. On the whole these results suggest a specially active participation in fat metabolism by the lipins of the liver. Artom *et al.* [1937] have reached a similar conclusion from experiments in which radioactive P was used to trace the entry of  $H_3PO_4$  into lipins. The lower % D in the plasma lipins in comparison with those of the liver at the end of 6 hr., renders it unlikely that the liver lipins were not synthesized in the liver but merely taken up from the blood.

The D content of the adipose tissue was never very marked and this was to be expected because of the diluent effect of the comparatively large amount of adipose tissue fat in the animal compared with that (1.9 g.) which was administered.

The brain in all the experiments contained only traces of D in its lipin and glyceride fractions. This does not suggest any rapid exchange in this organ between its lipins and the blood fat. It confirms observations made by Hahn & Hevesy [1937], who found a definite but small uptake of labelled P by brain lipins an hour after the injection into rats of Na phosphate containing the radioactive isotope of phosphorus.

#### SUMMARY

1. Rats were fed with a fat containing 4-5 atoms % deuterium and the distribution of the "deutero-fatty acids" in "lipin" and "glyceride" fractions of liver, kidney, brain and blood determined 6, 10 and 24 hr. after.

2. After 6 hr. D was present in considerable amount in plasma glycerides, liver glycerides and liver lipins. There was much less in the lipid fractions of the kidney and plasma and the D was only present in traces in brain and adipose tissue.

3. The D in the liver glycerides decreased more rapidly in 24 hr. than that of the liver lipins. In the lipid fractions of kidney and brain there were no notable changes in % D between 6 and 24 hr.

4. The results suggest that liver lipins may play a very active part in fat metabolism.

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### III. THE CALCIUM REQUIREMENT OF OLDER MALE SUBJECTS

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It is now generally accepted that Ca is an important essential in nutrition. It is believed, moreover, that, in most countries of the world, large sections of the population would be more adequately nourished were they to ingest more Ca. This applies to Great Britain [Orr, 1937], India [McCarrison, 1932-3], U.S.A. [Sherman, 1920, 2], China [Kung & Yeh, 1937; Liu *et al.* 1937], and figures given by Rubner [1920] leave little doubt that it applies also both to Germany and to Japan.

Orr & Clark [1930] showed that the health of school children could be improved and that their growth could be accelerated by giving them a supplement of whole or separated milk. More recent experiments by Gaunt *et al.* [1938] indicated that the benefits resulting from the addition of milk and greenstuffs to poor-class diets were due mainly to the Ca and P which these additional foodstuffs contain. Aykroyd & Krishnan [1938] have found the health and growth rates of Indian school children to be improved by a daily ration of Ca lactate.

The food shortage in Germany and Austria during the world war showed very clearly the result of a widespread lack of Ca and P. Hundreds of cases of "hunger osteomalacia" occurred. Alwens [1919], after careful clinical investigation of 29 such cases, mostly old subjects, in Frankfurt, concluded that they were suffering from osteoporosis due to protein, Ca and P deficiency. Dalyell & Chick [1921] investigated over 100 cases and also thought "hunger osteomalacia" to be a nutritional disease of the skeleton. Hume & Nirenstein [1921] showed that the incidence of the disease was highest in winter when the diet was poorest and that there was a remarkable increase of incidence with age. Of 131 subjects examined 115 were over 40 years of age and one-third were between 60 and 70.

Such observations show the importance of the hypothesis of Meulengracht & Meyer [1936] who, after intensive investigation of 5 cases of senile osteoporosis, came to the conclusion that long-standing Ca deficiency was the main factor in the genesis of this disease. Meulengracht [1938] has again advanced this hypothesis in connexion with a case of senile osteoporosis produced by repeated purgation with  $\text{Na}_2\text{SO}_4$ . Leitch [1936-7] supports Meulengracht's opinion as to the relation of Ca deficiency to senile osteoporosis.

Before the effects of a long-standing deficiency of Ca can be properly evaluated it is necessary to ascertain the Ca requirement of older subjects. A decrease of requirement with age would offset, to some extent, the effects of low intake. It is also of importance to find out whether, after depletion of these elements, the older organism will store additional Ca and P in the same way as the young adult. The following balance experiments were therefore undertaken to decide these points.

## PLAN OF EXPERIMENTS

The experiments were all done in the metabolic ward of the Aberdeen Royal Infirmary. Balances were determined on 10 subjects, mostly old or middle-aged. A preliminary period on a low Ca diet was followed by one on a higher intake. A specially trained sister supervised the preparation and consumption of the diets and the collection of all excreta.

In all cases balances were determined every 3 days but the data for the first 3 days on the low Ca diet were not included in the results. Both low and high Ca periods lasted at least 9 days.

Duplicate diets were analysed for total Ca and P in 3-day lots; urine and faeces were similarly analysed every 3 days. The methods of analysis were, with minor modifications, those recommended in *Technical Communication No. 9* of the Imperial Bureau of Animal Nutrition [Godden, 1937], i.e. P was estimated by the method of Fiske & Subbarow [1925], and Ca by the method of McCrudden [1909; 1911]. Ca in urine was determined by direct precipitation. The urine was, if necessary, deproteinized prior to precipitation, acetic acid being used for the purpose. Ca in ashed diet and in ashed faeces was determined by precipitation of the oxalate in the presence of sodium acetate. Dry ashings preceded all P determinations; in the case of diet and faeces,  $\text{Mg}(\text{NO}_3)_2$  was added and with urine Ca acetate was employed. All ashings were completed in an electric muffle.

Male outpatients from the diabetes and obesity clinic of Dr A. Lyall were used; they varied in age from 32 to 70 and were kept in bed during the whole period of the experiment. Blood Ca and P, and serum phosphatase of all patients were within normal limits.

The dietary history of these patients for the previous 6 months was known, including their Ca and P intakes. The composition of the diet during the preliminary low Ca period was adjusted to a similar level. The diets used in the balance experiments consisted entirely of normal foodstuffs and the Ca and P contents in the second period were raised solely by addition of foods rich in these materials. In cooking the food, distilled water was used and all such water was included in the diet, for example in soups. Eggs were fed always without shells since some patients were found to eat egg-shells. Only on very few occasions did patients reject food; in all such cases a similar rejection was made from the duplicate diets. The composition of the two diets is given in Table I.

Table I. *Composition of diets I and II expressed as daily intakes*

	Diet I	Diet II
Calories	1493	1509
Protein	59	85
Carbohydrate	141	146
Fat	77	65
Ca	0.53	0.88
P	0.85	1.23
Ca/P ratio	0.62	0.72
Ca as % of dry wt. of diet	0.184	0.273
P as % of dry wt. of diet	0.294	0.381

## RESULTS

All the data relevant to the Ca and P retentions of the subjects investigated will be found in Table II. On the lower Ca level it will be seen that four of the subjects were in positive Ca balance and five in negative, while the remaining one

Table II. *Summary of balance experiment results*

Subject no.	Age of subject	No. of 3-day balances	Ca (mg. per day)					Balance
			Intake	Output				
				Urine	Faeces	Total		
1	53	7	510	130	383	513	- 3	
		1	819	157	532	689	+ 130	
2	45	5	491	141	370	511	- 20	
		4	861	206	539	745	+ 116	
3	66	3	503	192	382	574	- 71	
		3	869	195	605	800	+ 69	
4	53	3	503	199	251	450	+ 53	
		3	869	260.5	463.5	730	+ 140	
5	42	3	510	156	531	687	- 177	
		3	942	201	472	673	+ 269	
6	32	3	510	165	370	535	- 25	
		3	942	214	544	758	+ 184	
7	69	3	567	77	450	527	+ 40	
		3	896	100	505	605	+ 291	
8	65	3	567	65	281	346	+ 221	
		3	896	104	595	699	+ 197	
9	65	3	544	49	407	456	+ 88	
		3	849	77	660	737	+ 112	
10	40	3	544	542	136	678	- 134	
		3	849	544	293	837	+ 12	
Average of the 10 subjects			524	172	356	528	- 4	
			879	206	521	727	+ 152	

Subject no.	Age of subject	No. of 3-day balances	P (mg. per day)				
			Intake	Output			Balance
				Urine	Faeces	Total	
1	53	7	879	832	200	1032	- 153
		1	1210	960	256	1216	- 6
2	45	5	851	814	214	1028	- 177
		4	1286	987.5	283.5	1271	+ 15
3	66	3	839	746	245	991	- 153
		3	1201	807	365	1172	+ 29
4	53	3	839	836	135	971	- 132
		3	1201	1083	238	1321	- 121
5	42	3	840	833	242	1075	- 235
		3	1226	964	237	1201	+ 25
6	32	3	840	760	208	968	- 128
		3	1226	962	277	1239	- 13
7	69	3	845	737.5	229.5	967	- 122
		3	1311	883	249	1132	+ 179
8	65	3	845	630	193	823	+ 22
		3	1311	664	428	1092	+ 219
9	65	3	858	699	227	926	- 68
		3	1149	691	428	1119	+ 30
10	40	3	858	1015	125	1140	- 282
		3	1149	977	210	1187	- 38
Average of the 10 subjects			849	790	202	992	- 147
			1227	898	297	1195	+ 32

was in equilibrium. The average intake of the whole group while on the lower Ca diet was 524 mg. Ca per day, and the average output was 528 mg. per day, i.e. the subjects as a group were practically in equilibrium.

The P balances on the lower Ca diet were negative in every case except no. 8. The average P intake was 849 mg. and the average output 992 mg. per day.

At the higher Ca level all the subjects without exception went into positive balance. The average Ca intake of the group was 879 mg. per day and the average output 727 mg. per day. In all but one case 900 mg. Ca per day would certainly more than suffice to keep these subjects in Ca equilibrium. The time necessary to attain equilibrium at the higher level of intake is evidently considerably longer than the duration of the present experiments.

On the higher Ca intake the P intake was also higher and the average P balance of the group was positive. The average P intake was 1227 mg. per day and the average output 1195 mg. per day. Individually the subjects were all very nearly in equilibrium except no. 4 who was in a distinct negative balance and nos. 7 and 8 who were in strongly positive balance. At the higher level of P intake no. 4 was still losing P despite his having been in no way unusual at the lower level. At both levels of intake he was retaining Ca.

All the subjects excreted P chiefly in the urine at both intake levels, while Ca was excreted chiefly in the faeces. No. 10 was unusual in excreting much more Ca in the urine than in the faeces. On the lower Ca diet his output in the urine alone almost exactly equalled the total Ca ingested. This abnormality suggested further investigation which is now being carried out.

## DISCUSSION

The figure here obtained for the Ca requirement of older men agrees well with the estimate of 450 mg. per person per day given by Sherman [1920, 1]. It also agrees well with that of Leitch [1936-37] for adult, non-pregnant, non-lactating women; she found a requirement of 550 mg. per day. Brull [1936], from subjects who were in negative balance, arrived at a similar estimate, though it is doubtful if the output of a subject in negative balance is a true index of his requirement. The data of Kelly & Henderson [1929-30] seem to indicate retention at a level considerably lower than the one here found. As their subjects were negro prisoners in Africa these better retentions may have indicated that the skeletons of their subjects were partly depleted of Ca and P before coming under investigation, since Rottensten [1938] has shown that retention may be more efficient in such circumstances. The subjects had been subsisting for some time on the prison diet containing 300 mg. Ca and 2390 mg. P per day.

The P requirement of the older adults here considered is approximately 1200 mg. per day. This figure exceeds the estimate of 900 mg. given by Sherman [1920, 1]. Requirements of this order have, however, frequently been reported, as shown in the review of P metabolism by Forbes & Keith [1914]. Figures quoted by Orr & Clark [1930] are also of this order.

Both diets were of normal composition and it is of interest that the Ca/P ratio was so low in each case. This is, however, apparently usual in human diets since many diets determined by survey at the Rowett Research Institute have similarly low ratios. Little can be argued from the ratio in the present experiments since it is virtually the same in both diets.

It may appear strange that subjects who were apparently in equilibrium on the lower intake should have gone into marked positive balance when the intake was increased. This is almost certainly due to the fact that the lower level

represents the lowest for maintenance and allows no margin against unforeseen Ca loss. It had been noted that at this level an attack of diarrhoea might cause a considerable loss of Ca. A study of Table II shows that at the lower level there were as many negative as positive balances, while at the higher level they were all positive. This increase in or change to a positive balance probably represents the making good of small losses of Ca incurred over a considerable prior period.

The Ca requirements of older males are much the same as those of younger adults: the effect, therefore, of long subjection to a diet low in Ca is not offset by increasing age. The older organism likewise resembles the young adult in that it readily retains Ca and P after depletion. The possible bearing of these facts on the genesis of senile osteoporosis has been discussed in the introduction.

### SUMMARY

Ca and P balances determined on 10 older males are reported, and the possible bearing of the results on the aetiology of senile osteoporosis is discussed. The results indicate that Ca and P equilibrium may be attained with 520 mg. Ca and 1200 mg. P per day.

The writer is indebted to Dr A. Lyall, Clinical Chemist to the Aberdeen Royal Infirmary, for providing the clinical material, for supervising the management of the patients and for much advice; to Sister Stephen for her help in the accurate preparation of the diets and careful collection of the excreta; and to Dr J. T. Irving, Head of the Physiology Department of the Rowett Research Institute, for his help and criticism. This work was made possible by a whole-time grant from the Medical Research Council for which grateful acknowledgment is made.

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## IV. ENZYMIC METHODS FOR THE PREPARATION OF ARGININE AND ORNITHINE

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THIS paper deals with two examples of the application of enzymes to preparative purposes: (1) the use of "trypsin" in the preparation of arginine, and (2) the use of arginase with urease in the preparation of ornithine. The first, while not devoid of practical utility, is described chiefly as a demonstration of the existence of substantial amounts of free arginine in pancreatic digests. The second is offered as a wholly satisfactory method for the preparation of ornithine, possessing certain advantages over any hitherto described.

### *1. Isolation of arginine from pancreatic digests*

Some years ago Dauphinee & Hunter [1930] showed that digestion with commercial trypsin would split off, in a form accessible to the action of arginase, about 80 % of the total potential arginine of certain proteins. The question, whether the arginase-susceptible material thus liberated is arginine itself or an arginine complex, was at that time left open. Later (unpublished) observations by Mr J. W. Chambers and the writer have indicated that it is probably a mixture, in which demonstrably free arginine forms at the beginning a relatively small, but, as proteolysis proceeds, an increasingly large, proportion of the whole. When, after about two weeks of digestion, that whole has reached its maximum, as much as 80 or 90 % of it may be simple arginine.

This fact may be utilized in the preparation of arginine from protein by substituting for the usual acid hydrolysis the milder action of the pancreatic enzymes. The substitution has drawbacks, but also some advantages. Its drawbacks are (1) that, since the enzyme hydrolysis is incomplete, the yield of arginine is correspondingly reduced, and (2) that the need to separate the free arginine from arginine-containing complexes calls for a double precipitation with flavianic acid. The advantages are a simplification of the initial manipulations, and, more important, the avoidance of the partial racemization, which is produced by the long-continued action of boiling acids. The enzymic method leads, it will be shown, to a product of exceptional optical purity. How far this may compensate for the lower yield will depend upon circumstances.

The procedure is applicable to any protein which contains a relatively high proportion of arginine, and which at the same time is hydrolysable to a sufficient extent by the pancreatic enzymes. The detailed description which follows is adapted specifically to the case of gelatin.

250 g. air-dry gelatin are dissolved in 2500 ml. water at 37°. By appropriate addition of NaOH the solution is made definitely alkaline to phenolphthalein, and it is then treated with 10 g. of an active commercial trypsin suspended in about 50 ml. water. The mixture is covered with a layer of toluene and incubated at 37° for 15 to 20 days, i.e. until the "free" arginine, as determined by arginase, has reached a maximum.

The digest is now acidified with 5 *N* H<sub>2</sub>SO<sub>4</sub> (at the rate of 2 ml. per 100), boiled, filtered from coagulated protein, heated almost to the boiling point, and treated with 450 ml. of a 20 % solution of flavianic acid. After a day or two in the ice-chest the granular yellow precipitate of crude arginine flavianate is collected on a large fritted glass filter (porosity 3), and washed with a cold 0.1 % solution of flavianic acid in 0.025 *N* H<sub>2</sub>SO<sub>4</sub>.

For purification the flavianate is suspended in about 1800 ml. water, and brought into solution with the minimum amount (about 20 ml.) of 5 *N* NaOH. The solution is diluted to about 2500 ml., heated again just short of boiling, and treated with a mixture of 50 ml. 5 *N* H<sub>2</sub>SO<sub>4</sub> and 225 ml. (half the amount used in the first precipitation) of 20 % flavianic acid. In a minute or two the arginine flavianate begins to separate from the hot solution, this time in the form of large glistening iridescent orange-yellow leaflets. The mixture is allowed to cool slowly to room temperature, and is then set in the ice-chest for at least 24 hr. The precipitate is finally collected and washed in the same way as the first.

Precipitation with flavianic acid in the heat, as recommended, has several advantages. Not only does it yield the product in a form especially convenient for further manipulation, but it affords also a certain criterion of its purity; for if, under the conditions described, the flavianate assumes a granular form, it is almost certainly still contaminated.

From the purified arginine flavianate the arginine may be recovered by any of the standard methods, but the procedure adopted in this laboratory is the following. The flavianate is suspended in 500 ml. water and brought into solution with the minimum amount (about 12 ml.) of conc. NH<sub>3</sub>. The solution is diluted to about 800 ml. and treated with a definite excess of cold saturated baryta water. The precipitate of barium flavianate is filtered off under suction, and washed thoroughly with very dilute baryta. From the mixed filtrate and washings the barium is precipitated quantitatively with H<sub>2</sub>SO<sub>4</sub>, and the residual flavianic acid is removed by treatment with the minimum amount of norite. BaSO<sub>4</sub> and norite are filtered off together. The colourless filtrate is freed of NH<sub>3</sub> by evaporation to a small volume, is then neutralized exactly with HCl and is finally evaporated to dryness, yielding directly a crystalline mass of arginine hydrochloride. This product, weighing as a rule between 15 and 17 g., is purified by dissolving it in a little water, rubbing up the solution with small amounts of alcohol until crystallization sets in, adding then about two more volumes of alcohol, and cooling overnight in the ice-chest.

In several experiments with this procedure the final yield of pure arginine hydrochloride has varied between 13.5 and 15.6 g. A representative sample gave by Kjeldahl 26.48 % N (C<sub>6</sub>H<sub>14</sub>O<sub>2</sub>N<sub>4</sub>·HCl requires 26.60 %), and left on incineration less than 0.1 % of ash. In the presence of seven extra molecules of HCl it had  $[\alpha]_D^{25} = 22.19^\circ$  ( $c = 11.47$ ;  $l = 2.2$  dm.;  $\alpha = +5.60^\circ$ ). The highest value hitherto recorded for the specific rotatory power of arginine hydrochloride under the conditions specified is  $+21.95^\circ$  [Hunter & Morrell, 1922; Hunter, 1929]. The product of the enzymic method of preparation was therefore *l*(+)-arginine of at least as great a degree of optical purity as has so far been attained. The experiments gave no indication whatever of the production of inactive arginine, such as has been described by Kutscher [1899; 1901] as occurring during the tryptic digestion of fibrin.

The gelatin used in the present work contained, according to the arginase method, 7.73 g. of potential arginine per 100 g. (air-dry). The yields of recrystallized hydrochloride mentioned above represent therefore only from 58 to 67 % of the possible maximum and roughly three-fourths of the amount recoverable

from a total hydrolysate by the excellent method of Cox [1928]. Of course, it has to be remembered that in the enzyme hydrolysates only 80 % or so of the total arginine had been so far liberated as to be accessible to the action of arginase [Dauphinee & Hunter, 1930]. Of this fraction the proportion finally accounted for as pure hydrochloride is from 73 to 84 %. This result was attained by procedures which, on the one hand, would be unlikely to bring about more than a minimal hydrolysis of arginine peptides, and which, on the other, certainly involved some losses. It seems therefore not unreasonable to conclude that in the final stages of pancreatic digestion at least 80 or possibly as much as 90 % of the arginase-susceptible material is actually free arginine.

The literature contains a few previous reports of the isolation of arginine in substance from pancreatic digests [Kossel & Mathews, 1898; Kutscher, 1898; 1899; 1901]; but the yields (never exactly stated) in these earlier experiments appear to have been relatively small.

## 2. Preparation of ornithine

The methods heretofore proposed for the preparation of ornithine from arginine have varied with respect to (1) the agent employed to hydrolyse the arginine, (2) the procedure followed in separating the ornithine from urea and from the hydrolysing reagent and (3) the form in which the ornithine has been finally isolated. For the hydrolysis use has been made of  $\text{Ba}(\text{OH})_2$  [Schulze & Winterstein, 1898; Bergmann & Zervas, 1926; Kurtz, 1938],  $\text{NaOH}$  [Boon & Robson, 1935] and arginase [Kiesel, 1911; 1922; Felix & Röthler, 1925; Vickery & Cook, 1931]. To separate the ornithine Schulze & Winterstein [1898] employed benzylation, Bergmann & Zervas [1926] and Boon & Robson [1935] condensation with salicylaldehyde, and the other authors (Kurtz excepted) precipitation with phosphotungstic acid. As a rule the base has ultimately been converted into the hydrochloride; but Kiesel [1911] isolated it as carbonate, Felix & Röthler [1925] and Vickery & Cook [1931] as picrate, Kurtz [1938] as sulphate.

The earlier methods, whatever combination of procedures they represented, gave very unsatisfactory yields. Schulze & Winterstein [1898] appear to have obtained from arginine at most 30 % of the theoretical amount of ornithine; and no better result was claimed for any of the alternative procedures published prior to 1931. Parenthetically it may be mentioned that in 1929 Mr H. B. Collier, working under the writer's direction, obtained ornithine hydrochloride in yields up to 45 % by the action of arginase upon arginine, followed by condensation of the product with salicylaldehyde in the presence of  $\text{Ba}(\text{OH})_2$ . Collier's results were left unpublished because of uncontrollable irregularities encountered in the application of the salicylaldehyde method. Vickery & Cook [1931] also had difficulties with this method, but by the use, under special conditions, of phosphotungstic acid obtained from an arginase digest ornithine (as dipicrate) corresponding to 55 % of the arginine taken.

Really satisfying yields ("approximately theoretical") were first attained by Boon & Robson [1935], who recommended hydrolysis (of carbamido-arginine) with 20 %  $\text{NaOH}$ , separation of ornithine as the barium salicylidene derivative, and conversion of the latter into the hydrochloride. In the yet more recent method of Kurtz [1938] carbamido-arginine is treated with baryta under conditions which not only split it into ornithine and urea, but which also ensure the destruction of the latter. Since the baryta itself is readily removed, preliminary separation of the ornithine can be dispensed with, and it is precipitated directly (as sulphate) by alcohol. Yields range from 76 to 82 %.



The method of Kurtz is the simplest yet proposed; but, like all methods depending on prolonged hydrolysis by alkalis, it has the drawback of yielding a product which is either partially or completely racemized. If the natural dextro-rotatory ornithine is required, it is desirable, if not imperative, to resort in the first place to enzymic hydrolysis, and to restrict to the necessary minimum any subsequent exposure of the product to the action of alkali. Now for the successful application of the enzymic method two points are of practical importance. In the first place, in order to effect a complete hydrolysis of the arginine (which previous enzyme methods have evidently failed to do) it is necessary to use a large amount of arginase—about 1000 units [Hunter & Dauphinee, 1930] per g. arginine. In the second, it is obviously desirable to introduce along with the enzyme as little extraneous and inert material as possible. From this point of view a crude glycerol extract of liver is a quite unsuitable reagent. Later there is described the preparation from aqueous liver extracts of a concentrated arginase solution containing in 1 ml. from 250 to 380 units of the enzyme but only a fraction of the solid material with which that enzyme was originally associated. Under appropriate conditions from 2.5 to 4 ml. of such a solution amply suffice for the complete hydrolysis of 1 g. arginine, yet introduce little that cannot afterwards be removed by heat coagulation. There is therefore a possibility of preparing digests, which contain hardly anything in solution but an ornithine salt and urea.

It was thought at first that the separation of these two substances from one another might be accomplished in a very simple way by taking advantage of their greatly differing solubilities in alcohol. Had this proved possible, the steps necessary for the isolation of ornithine would have been few and straightforward. Unfortunately attempts to precipitate ornithine from the concentrated protein-free digest with alcohol, or to recrystallize from alcohol the solid residue left on evaporation, gave disappointingly small yields of a product which was always contaminated with urea. It remained necessary, therefore, either (a) to precipitate the ornithine first as phosphotungstate or other insoluble compound or (b) to destroy the urea. Since one of the objects sought was the elimination of the usual intermediate precipitation, the alternative adopted was the second; and in order to keep the whole method an enzymic one the agent chosen for the destruction of urea was urease. This can be quite conveniently applied in combination with arginase, so that the decomposition of urea may proceed *pari passu* with the production of ornithine. The alkaline reaction rapidly produced by the development of ammonium carbonate favours the action of the arginase without unduly depressing that of the urease. When the process is complete, both the ammonium and the carbonate ions can be got rid of by distilling the mixture with an excess of baryta. The barium having been in its turn removed, one is left with a solution of ornithine (or an ornithine salt), which can be freed from residual (mostly inorganic) impurities in the ordinary way.

The methods of preparation described below are based on the principles thus outlined.

#### (A) *Enzyme reagents required*

**Arginase.** A crude arginase extract is prepared according to the method of Hunter & Dauphinee [1930] with the single change that water is substituted for 75 % glycerol. One volume of this extract is mixed with 1.2 volumes of acetone. The flocculent precipitate is separated by centrifuging, and extracted with half the original vol. of water. Any insoluble material is filtered off, and the filtrate is treated again with 1.2 vol. of acetone. The second precipitate is taken up in

one-fourth of the original volume, and to this final solution cobalt chloride is added in the proportion of one drop of a 1 % solution for each 10 ml. The activity of the solution is determined by the method of Hunter & Dauphinee [1930]. It should contain the least 200, preferably between 300 and 400, units of arginase per ml.

*Urease.* 10 g. jack-bean flour are shaken for 10 min. with 100 ml. of 30 % alcohol, and the mixture is then filtered.

(B) *Preparation of ornithine monohydrochloride from arginine hydrochloride*

To a solution of 10 g. arginine hydrochloride in 250 ml. water there are added (1) the prescribed arginase solution in amount sufficient to provide 8000 units of the enzyme, and (2) 10 ml. of the urease solution. The mixture, protected by toluene, is incubated at 37°. At first faintly acid, it rapidly develops an alkaline reaction and a strongly ammoniacal odour. After 4 or 5 days it is made just acid to Congo red with 5 *N* H<sub>2</sub>SO<sub>4</sub> (about 18 ml.), and is boiled to coagulate proteins. (Acidification may, if it is thought worth while, be preceded by a preliminary distillation *in vacuo* with alcohol; this effects the removal of a considerable proportion of the NH<sub>3</sub>, and reduces correspondingly the subsequent consumption of H<sub>2</sub>SO<sub>4</sub> and baryta.) The mixture is cooled, and treated with a considerable excess (about 225 ml.) of saturated baryta water. The precipitate of coagulated protein and insoluble Ba salts is filtered off and washed with dilute baryta water. Filtrate and washings are transferred to a large Claisen flask, and freed from NH<sub>3</sub> by distillation *in vacuo* at a temperature not exceeding 50°. Frothing, when it occurs, is controlled by liberal additions of alcohol, repeated, as often as may be necessary, until the evolution of NH<sub>3</sub> comes to an end. The distillation residue should then be negative to Nessler's reagent. If it is not, more baryta is added, and the distillation continued.

When one is certain that the ammonia has been completely removed, the residue is freed exactly from barium by H<sub>2</sub>SO<sub>4</sub>, and filtered. It should now be neutral or only slightly alkaline, and give no more than a feeble Sakaguchi reaction for arginine. Having, if necessary, been made exactly neutral with HCl, it is concentrated on the water bath to about 75 ml., decolorized by heating with a generous addition of norite, filtered, and evaporated to dryness. If the product becomes seriously discoloured during evaporation, the treatment with norite is repeated. There is thus obtained a crude ornithine monohydrochloride, nearly white, but contaminated as a rule with a considerable quantity of inorganic salts.

For its purification the crude material is dissolved, by prolonged boiling under a reflux condenser, in the smallest possible volume of 75 % alcohol. If necessary, the hot solution is rapidly filtered through a fritted glass filter. Crystallization begins almost immediately. After a day in the cold chamber the crystals are collected, and washed with a little 95 % alcohol. This first crop should weigh about 6 g., and should not contain more than 0.3 % ash. A second crop of equal purity is obtained by evaporating the mother liquors to dryness and again recrystallizing the residue from 75 % alcohol. In this way the yield may be increased to 7 g. or more.

The results of two experiments with this method, including the N (Kjeldahl) and ash contents of the products, are given in Table I. The lower yield of the first experiment is accounted for by the fact that in this, an early one, only one crop of crystals was collected. With a little experience it is easy to reproduce the improved results of the second.

(C) *Preparation of ornithine hydrochloride or sulphate from protein*

The success of the enzymic method as applied to arginine itself suggested that it might be possible by similar means to isolate ornithine directly from any protein hydrolysate reasonably rich in arginine. In this way one would not need first to prepare (or procure) pure arginine. This idea, in its first shape, was frustrated by the difficulty of separating the ornithine from the many other substances in the arginase-treated hydrolysate. In a modified form it proved to be entirely feasible. Neither arginase nor urease is inactivated by flavianic acid. All that is necessary therefore to obtain ornithine from a protein is to hydrolyse the latter, precipitate the liberated arginine once with flavianic acid, and treat the arginine flavianate in the same way as one would the hydrochloride. When this is done, the baryta used to liberate  $\text{NH}_3$  serves also to remove the bulk of the flavianic acid. The ornithine may be isolated either as hydrochloride or as sulphate. In detail the procedure is as follows:

A convenient weight of some appropriate protein (say 200 g. gelatin or 50 g. protamine) is hydrolysed by boiling for 12 hr. with a ten-fold quantity of 20 % HCl. The hydrolysate is freed, as far as may be possible, from HCl by concentration *in vacuo* to a thick syrup. It is then taken up in water, neutralized to Congo-red with 40 % NaOH, decolorized with norite, filtered, and diluted to approximately its original volume. If its estimated concentration of arginine is then much greater than 1 % (as would be the case with a protamine hydrolysate) it may with advantage be diluted even further. The solution is now heated to the point of boiling, and treated with 20 % flavianic acid. The quantity of this to be used is calculated at the rate of 20 ml. for each g. of expected arginine. The mixture having been cooled, first to room temperature and then for at least 24 hr. in the ice-chest, the flavianate is collected under suction on a large fritted-glass funnel, washed, suspended in several vol. of water and brought into solution with the minimum amount of  $\text{NH}_3$ . The solution is diluted until it contains approximately 3 % of arginine, and is then digested with arginase and urease. The quantities of these are calculated so as to supply 1000 units of arginase and 1.4 ml. of urease solution for each g. of arginine believed to be present. Digestion is continued, in the presence of toluene, for 4 days at 37°.

With or without a preliminary vacuum distillation (see Section B) the strongly ammoniacal digest is neutralized with 5 N  $\text{H}_2\text{SO}_4$  and boiled. Additional acid is added sufficient to ensure the exact coagulation of the proteins. The cooled mixture is treated with an adequate excess of saturated baryta water, and filtered. The precipitate is washed with dilute baryta. Filtrate and washings are combined, and freed from  $\text{NH}_3$  by vacuum distillation just as in the preparation from arginine.

The further treatment of the material depends upon whether it is decided to isolate the ornithine as sulphate or as hydrochloride.

(a) *Ornithine monohydrochloride.* To obtain the hydrochloride the solution left in the distilling flask is filtered (if necessary), exactly freed from Ba with  $\text{H}_2\text{SO}_4$ , shaken with enough norite to remove residual flavianic acid, filtered, neutralized to litmus with HCl, and concentrated *in vacuo* to about 100 ml. The colour which usually develops during this concentration is removed by boiling with norite, and the filtered solution is evaporated to dryness. The crude hydrochloride is finally recrystallized from 75 % alcohol in the manner described already in Section B.

(b) *Ornithine monosulphate.* To obtain the sulphate the solution is neutralized exactly to litmus with  $\text{H}_2\text{SO}_4$ , a step which incidentally frees it from Ba. It is

then decolorized, filtered, concentrated, again decolorized—all in the same way as with the hydrochloride—and finally evaporated on the water bath, not to dryness, but only until crystallization begins. When it has cooled, it is stirred vigorously with successive small additions of absolute alcohol. This brings about the separation of more sulphate, at first in the form of milky globules, presently coalescing into a heavy oil. As the operation is continued, the oil gradually solidifies, the crystals get harder and harder, and finally the whole is converted into a heavy crystalline powder. More alcohol is added, and the mixture is left overnight in the ice-box. The alcohol is then poured off, and the crystals are washed, by stirring and decantation, 3 times with cold 95 % alcohol. The crystals are allowed to dry in the air, being stirred frequently the while, in order to prevent them from forming heavy crusts or sticking to the sides of the dish. They are then dried further in the desiccator and finally at 110°.

It will be gathered from this prescription that the physical properties of the sulphate differ from those of the hydrochloride. Kossel & Weiss [1909] describe an optically inactive ornithine sulphate which can be readily recrystallized from hot 60 % alcohol. The enzymically prepared sulphate, which is dextrorotatory, cannot be conveniently recrystallized in this way. Heated with either 60 or 75 % alcohol it yields for the most part only an oil. Recrystallization from 85 % alcohol is possible, but requires an impracticably large volume of solvent. Collected and dried on a filter in the usual way, the crystals form a hard cement-like cake, which can hardly be detached or broken up even with a steel spatula. By the methods of crystallization, washing and drying described above one escapes these several inconveniences.

The preparation of the sulphate involves fewer and simpler manipulations than that of the hydrochloride, and its yield is at least equally good. Its sole disadvantage is that the product may contain a rather high proportion—up to 3.6 % of ash. This consists mainly of calcium sulphate. It can be eliminated by repeated recrystallization from water, but the ornithine salt itself is so soluble, that purification by such means would be highly unprofitable.

The methods described have been applied with success to gelatin and to protamine, and Table I gives the data of three experiments with these materials. Two (with gelatin) exemplify the preparation of ornithine sulphate, the third (with protamine) that of the hydrochloride. The gelatin used was of the same brand as that employed in Part I for the preparation of arginine, so that 100 g. (air-dry) were capable of yielding 7.48 g. of ornithine monohydrochloride or 8.04 g. of ornithine monosulphate. The protamine was derived (as a sulphate) from an unidentified species of Pacific Coast salmon (*Oncorhynchus*). Its degree of purity was unknown. Analysis showed that it contained 11.9 % water, 18.5 %  $\text{H}_2\text{SO}_4$  and 19.9 % N. On ignition it left 5.54 % ash, largely sulphate. The arginase method indicated that arginine accounted for 77.2 % of the total N—much less than the 89.3 % found by Waldschmidt-Leitz *et al.* [1931] for purified salmine. According to these data 100 g. of the air-dry sulphate contained 47.75 g. of potential arginine. The equivalent amount of ornithine monohydrochloride is 46.2 g. The high arginine content of protamine makes it a particularly remunerative starting material for the preparation of ornithine.

On a review of the figures in Table I it may be seen that, whether one starts from arginine itself or from protein, the enzymic method is capable of yielding from 80 to 90 % of the theoretical amount of ornithine. With respect to nitrogen content the product, whether in the form of sulphate or of hydrochloride, shows a high degree of purity, and only the sulphate contains more than a trace of inorganic impurity.

Table I

	Arginine hydrochloride		Gelatin		Prota- mine sulphate
Weight taken, air-dry (g.)	—		200		50
Weight taken, ash- and water-free (g.)	10.00		175.6		41.3
Arginine content (g.)	8.27		15.5		23.9
Ornithine monohydrochloride corresponding (g.)	8.00		—		23.1
Ornithine monosulphate corresponding (g.)	—		16.1		—
	(1)	(2)	(1)	(2)	
Ornithine monohydrochloride recovered (g.)	6.00	7.17	—	—	18.7
Ornithine monosulphate* recovered (g.)	—	—	14.4	14.0	—
Percentage yield*	75	90	90	87	81
Ash, % of product	0.28	0.10	3.63	3.38	0.04
Nitrogen,* % of product	16.42	16.48	15.47	15.20	16.52
Nitrogen, % of product, theoretical	16.62		15.47		16.62

\* On an ash-free basis.

Determinations of specific rotatory power were made (1) on the monosulphate No. 1 from gelatin, (2) on the monohydrochloride from protamine, (3) on the dihydrochloride, prepared by adding the calculated amount of HCl to a solution of the monohydrochloride. The details and results of the measurements made are given in Table II. It should be stated that the first measurement on the monohydrochloride was made before, the others after, a single recrystallization from alcohol. This recrystallization, it will be seen, did not increase the specific rotation. The first and second measurements reported on the sulphate were made after, respectively, one and two recrystallizations from water. The product of the first recrystallization still retained 0.90% ash; that of the second had only 0.16%. The specific rotations of the two are not significantly different. The original sulphate, with 3.6% ash, gave a value rather lower than that shown in Table II. Whether this should be attributed to a racemic admixture or to other impurities is uncertain.

Table II. *Specific rotations of ornithine salts*

Salt of ornithine	<i>t</i>	<i>l</i>	<i>c</i>	$\alpha$	$[\alpha]_D$ calculated	
					For the salt	For ornithine
Monohydrochloride	23°	2.2	22.37	+4.98°	+10.13°	+10.08°
"	23	2.2	21.99	+4.85	+10.03	
"	23	2.2	5.496	+1.33	+11.00	
"	25	2.2	5.693	+1.37	+10.94	
Dihydrochloride	23	2.2	13.37	+5.11	+17.36	+26.96
"	25	2.2	10.60	+4.01	+17.20	+26.71
"	23	2.2	5.300	+1.95	+16.7	+25.9
Monosulphate	22	2.2	6.070*	+1.15	+ 8.61	+11.9
"	22	2.2	5.864*	+1.13	+ 8.76	

\* On an ash-free basis

As far as the mono-salts of ornithine are concerned, the only previous determinations of optical activity are that of Vickery & Cook [1931], who found for the enzymically prepared monosulphate  $[\alpha]_D^{20} = +8.4^\circ$ , and that of Dirr & Späth [1935], who give for the monohydrochloride  $[\alpha]_D^{20} = +12.12$ . For the first of these salts the values here exhibited are appreciably higher, for the second quite definitely lower. The discrepancies may be more apparent than real, for neither Dirr & Späth nor Vickery & Cook have recorded concentrations or other relevant details. In any case the lower values now assigned to the monohydrochloride are supported by the results for the dihydrochloride, into which it was

converted. For this salt there are on record three earlier determinations—that of Schulze & Winterstein [1901], who found  $[\alpha]_D = +16.8^\circ$  ( $c=5.06$ ;  $t=10^\circ$ ), that of Kossel & Dakin [1904], who give  $+16.6^\circ$  (no details), and that of Bergmann & Zervas [1926], who give  $+16.5^\circ$  ( $c=4.72$ ;  $t=19^\circ$ ). With these figures the present value  $+16.7^\circ$  (for  $c=5.30$  and  $t=23^\circ$ ) is in satisfactory agreement. As far, therefore, as the data enable one to judge, the enzymically prepared material was pure *l*(+)-ornithine hydrochloride.

The specific rotatory power of free ornithine is given by Vickery & Cook (1931) as  $+11.5^\circ$ . From the figures in Table II it may be seen that the rotation increases as the base combines with increasing proportions of acid, and that the effect of HCl in this respect is greater than that of  $H_2SO_4$ . It is further evident that both for the mono- and the di-hydrochloride specific rotatory power varies more than a little with concentration. In all these respects the behaviour of ornithine shows, quantitatively as well as qualitatively, a striking resemblance to that of arginine [Gulewitsch, 1899].

#### SUMMARY

Methods are described which exemplify (1) the use of "trypsin" in the preparation of arginine (as hydrochloride) from protein, and (2) the combined use of arginase and urease in the preparation of ornithine (as hydrochloride or sulphate) from arginine or from protein. The methods for ornithine give yields up to 90 % of the theoretical. The products are shown to possess a high degree of optical purity. The specific rotatory power of *l*(+)-ornithine monosulphate (in 6 % concentration) is  $+8.7^\circ$ , while that of the monohydrochloride varies from  $+10.08^\circ$  (at 22 %) to  $+11.0^\circ$  (at 5.5 %), and that of the dihydrochloride from  $+17.36^\circ$  (at 13 %) to  $+16.7^\circ$  (at 5 %).

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# V. THE MANIFOLD ACTIVITY OF TESTOSTERONE DIPROPIONATE AS COMPARED WITH THAT OF TESTOSTERONE PROPIONATE IN GONADECTOMIZED RATS

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ENOL esters of male sex hormones were first prepared by Ruzicka & Fischer [1936]. Miescher *et al.* [1937] have prepared several new members of this group, and have studied the effects of some of the most important testosterone compounds on capon's comb growth and on the weight of the seminal vesicles and prostate of rats. In a 10-day test the enol esters of testosterone increased the weight of the seminal vesicles less than did testosterone propionate, but with some of the diesters, in particular the dipropionate, the effect lasted much longer.

From the clinical point of view, preparations with more prolonged activity have evident advantages. Therefore the activity of one of the most interesting of the enol esters (according to Miescher *et al.* [1937]), namely, testosterone dipropionate, on weight and histological structure of the organs has been studied in this paper and compared with that of the propionate which is at present used almost exclusively in clinical practice. Testosterone propionate has already been studied by us [Korenchevsky, 1937, 1; Korenchevsky & Dennison, 1937; Korenchevsky *et al.* 1937; Korenchevsky *et al.* 1937, 1 and 2; Korenchevsky & Hall, 1937].

## *Technique*

The pure crystalline hormones were supplied by Messrs Ciba Ltd., and were dissolved in sesame oil. Each dose was contained in 0.2 ml. of oil, and was injected daily into male or female rats, gonadectomized at the age of 22–24 days, i.e. before sexual maturity. The injections were continued in males for 23 days and in females for 21 days. The injections into the rats of groups “+ 10 days rest” (see Tables I and III) were commenced 10 days earlier than in the other groups, and these animals were not injected for the last 10 days before killing. In all the other groups the rats were killed on the day following the last injection. In this way the final age of all the rats in any one litter was the same.

The final age of the *female* rats was about the same in all groups (average 69 days). A large number of litters of *male* rats was used for the experiments, the final age varying in the different litters from 74 to 106 days.

Since in castrated control rats the average weights of some of the organs vary significantly according to age, for the sake of a more accurate comparison two average weights are given for these organs in the group “control castrated rats” (Table I, col. II): (1) upper figure—average age 96 days, (2) lower figure—average age 78 days. For the other organs of rats in this group, the general average age was 88 days. The average age of the normal control rats was 95 days, there being no normal controls to the younger rats.

Table I. *Effect on actual weights of organs of castrated rats of testosterone dipropionate alone or in combination with oestradiol dipropionate*

	I	II	III	IV	V	VI	VII	VIII	IX	X
	Control rats injected with oil		Castrated rats injected with							
			Testosterone dipropionate							
			50 µg.		150 µg.		500 µg.		1500 µg.	
			Normal		Castrated		150 µg. + 10 days' rest		500 µg. + 10 days' rest	
Organs										
Seminal vesicles (mg.)	952	12	163	528	333	1026	1022	1197	51	601
Prostate (mg.)	853	65	330	624	557	963	1135	1028	86	677
Penis (mg.)	348	81	218	282	280	327	356	316	71	271
Preputial glands (mg.)	135	37	95	117	119	140	119	128	43	106
Adrenals (mg.)	58	74	71	55	54	58	52	56	59	78
Hypophysis (mg.)	9.5	10.6	12.7	11.6	11.3	12.3	11.3	9.3	20.7	21.8
Thymus (mg.)	349	472	530	477	490	188	228	142	309	242
Liver (g.)	12.37	10.48 9.22	11.74	13.06	12.24	11.24	12.07	10.05	9.16	9.43
Kidneys (g.)	2.32	1.83 1.61	1.96	2.29	2.06	2.32	2.23	1.92	1.85	2.10
Heart (mg.)	1047	920 747	984	966	956	1052	1018	855	756	896
Retropitoneal fat (g.)	13.0	11.1	10.2	11.9	10.1	11.2	13.5	8.0	8.4	11.6
Final body weight (g.)	342	310 275	313	310	305	329	336	254	245	290
Gain in body weight (g.)	116	100 132	155	160	148	104	99	115	77	86
No. of rats in group	6	7	5	8	8	4	4	4	4	4
Final age (days)	95	96 78	78	80	80	104	99	77	96	82



For economy of space, the data per unit of body weight have not been tabulated, but will where necessary be referred to in the text.

A detailed account of the results of histological investigation will be given elsewhere, only some preliminary statements being included here.

Other details of the experimental technique can be found in our previous papers.

#### *Castrated male rats*

The experiments were performed on 59 rats, divided into 11 groups as shown in Table I (groups I–X) and Table II (group XI). Groups XII and XIII in Table II contain additional data taken from our previous experiments on 8 rats [Korenchevsky *et al.* 1937, Table I].

Table II. *Effect on actual weights of organs of castrated rats of testosterone propionate in doses comparable with those of testosterone dipropionate in Table I*

Organs	XI	XII	XIII
	Rats injected with testosterone propionate		
	50 $\mu$ g.	500 $\mu$ g.	500 $\mu$ g. + 10 days
Seminal vesicles (mg.)	417	1228	284
Prostate (mg.)	507	1248	614
Penis (mg.)	256	301	276
Preputial glands (mg.)	103	154	118
Adrenals (mg.)	60	59	59
Hypophysis (mg.)	11.4	12.5	13.3
Thymus (mg.)	502	219	291
Liver (g.)	10.49	12.1	11.52
Kidneys (g.)	1.79	2.03	1.84
Heart (mg.)	864	932	927
Retroperitoneal fat (g.)	9.8	12.2	13.1
Final body weight (g.)	288	316	318
Gain in body weight (g.)	144	122	116
No. of rats in group	5	4	4
Final age	74	89	89

#### *Sex organs*

Injections of 500  $\mu$ g. of testosterone dipropionate daily (Table I, group VI) cause complete return to normal size, weight and histological structure of the seminal vesicles, prostate, penis and preputial glands, and this effect remains unchanged, or is even increased, 10 days after the last injection (group VII). Injections of 150  $\mu$ g. are unable to restore the size and weight of these glands completely to normal, and the restorative effect obtained in the seminal vesicles, and less so in the prostate, begins to decrease during the period without injections, the weights of the penis and preputial glands remaining unchanged. Increasing the daily dose to 1500  $\mu$ g. (group VIII) does not produce a significantly greater effect than that obtained with 500  $\mu$ g. (groups VI and VII). Simultaneous injections with oestradiol dipropionate (group X) show only a very weak co-operative effect (as judged by weight) on the seminal vesicles and prostate.

*Comparison with testosterone propionate.* While with the small dose of 50  $\mu$ g. (Table I, group III and Table II, group XI) the activity of the dipropionate is considerably less than that of the propionate, this difference is much less pronounced with the medium dose of 500  $\mu$ g. (Table I, group VI and Table II, group XII).

Supernormal seminal vesicles (1441 mg. average) are produced by 1400  $\mu$ g. of the propionate [Korenchevsky *et al.* 1937, Table I, col. IV] but not by 1500  $\mu$ g. of the dipropionate (this paper, Table I, group VIII). Thus the dipropionate is weaker than the propionate, although the difference is considerable only with the small doses.

A more important difference is the fact that the effect of 500  $\mu$ g. of the dipropionate is maintained or even increased 10 days after the injections have ceased (Table I, group VII), and even with 150  $\mu$ g. there is a considerable decrease only in the seminal vesicles (group V) while the effect of all doses of the propionate is very considerably decreased by the tenth day [Korenchevsky *et al.* 1937; see also Table II of this paper, groups XII and XIII].

The dipropionate appears to have a slightly better co-operative activity with oestradiol, since with the propionate [Korenchevsky & Dennison, 1937] an effect was recorded only on the seminal vesicles.

### *Other organs*

The changes produced by the dipropionate on the weights of other organs are similar to, or even greater than, those caused by the propionate. The effects are maintained for at least 10 days with both hormones.

*Adrenals.* The hypertrophied "castration" adrenals return to normal size and weight (except with the lowest dose) this restoration being explained by a decrease in the width of the cortex. Only the largest dose of 1500  $\mu$ g. causes considerable depletion of lipoid granules in the cortex.

*Thymus.* Involution, delayed in castrated animals, returns to or exceeds the normal level (as shown by weight and histologically) with medium and large doses of the hormone.

*Liver, kidney and heart.* The weights (actual and per unit body weight) of these organs, decreased after castration, return to or towards normal. Since histologically nothing abnormal (liver, heart) or a slight increase of some tubules or their cells (kidneys) was found, the enlargement obtained should be considered as a stimulation of these organs by the injections.

*Hypophysis.* It is difficult to decide how much significance should be attached to the slight enlargement of the hypophysis observed in most rats injected with all but the largest dose of the hormone. A certain amount of variation was present in all groups and the results obtained with the largest dose were rather indefinite. Oestradiol dipropionate, as usual, produced hyperplasia, which the simultaneous injection of testosterone dipropionate, in the dose used, did not prevent (Table I, groups IX and X).

*Thyroids, parathyroids, pancreas and spleen* did not show any changes.

*Fat deposition and gain in body weight.* There appeared to be a more pronounced gain in body weight with small doses of the dipropionate (groups III-V), but no definite changes in fat deposition, so that the increase cannot be explained by accumulation of fat in the body. 500  $\mu$ g. produced no definite change (groups VI and VII), while with 1500  $\mu$ g. (group VIII) both fat deposition and also gain in body weight were slightly decreased. The dipropionate, therefore, appears to have some effect on metabolism.

*Simultaneous injections of oestradiol dipropionate* (group X) neutralize the effects of the male hormone on the adrenals and also prevent more or less its stimulating activity on liver, heart and gain in body weight, but not the enlargement of the kidney (groups IX and X). Co-operative activity of the two hor-

mones is shown in the acceleration of the involution of the thymus. Similar results have been obtained previously after the addition of oestradiol to testosterone propionate [Korenchevsky & Dennison, 1937].

*Ovariectomized females*

Experiments were performed on 36 rats belonging to 8 litters and divided into 8 groups as shown in Table III.

Table III. *Effect on actual weights of organs of spayed female rats of testosterone dipropionate alone or in combination with oestradiol dipropionate (and compared with the effect of testosterone propionate)*

	I	II	III	IV	V	VI	VII	VIII
	Spayed rats injected with							
	Control rats		Testosterone dipropionate		Oestra- diol dipro- pionate		Oestra- diol dipro- pionate	Testo- sterone
	Normal	Spayed	150 $\mu$ g.	150 $\mu$ g. + 10 days rest	1500 $\mu$ g.	6 $\mu$ g.	6 $\mu$ g. + testo- sterone dipro- pionate 150 $\mu$ g.	pro- pionate 1500 $\mu$ g.
Uterus (cervix + horns, mg.)	359	31	113	63	246	212	245	242
Cervix of uterus (mg.)	103	13	48	29	111	92	109	114
Vagina (with clitoris, mg.)	241	168	280	259	410	240	337	338*
Preputial glands (mg.)	97	66	126	95	176	66	109	255
Adrenals (mg.)	58	68	48	42	50	69	68	62
Hypophysis (mg.)	11.8	11.0	9.6	7.7	7.8	21.6	18.5	11.8
Thymus (mg.)	382	590	445	487	147	257	257	87
Liver (g.)	8.03	8.45	9.68	10.32	8.69	8.58	8.55	8.73
Kidneys (g.)	1.42	1.51	1.69	1.71	1.74	1.77	1.89	1.76
Heart (mg.)	665	708	765	750	744	689	681	764
Retroperitoneal fat (g.)	9	13	13	12	9	8	9	5
Final body weight (g.)	191	236	243	240	225	188	205	213
Gain in body weight (g.)	70	95	108	95	82	56	69	53
No. of rats in the group	9	8	4	3	3	3	4	2

\* In group VIII the vaginal weight is given without clitoris.

The data in group V (testosterone dipropionate) are compared with those given in group VIII, from rats injected with testosterone propionate (1500  $\mu$ g.). The testosterone compounds were injected daily and the oestradiol dipropionate three times per week in the doses given in the table.

*Sex organs.* The restorative effect on the sex organs of the spayed rats was very pronounced, and similar to that of the propionate (compare groups V and VIII). The weight of the vagina after injections of both 150 and 1500  $\mu$ g., especially the latter, exceeded that of the normal control, but complete recovery of the uterus was not obtained even with the large dose. The duration of the effect of the injections was not studied, except in the case of the small dose (150  $\mu$ g., group IV), when the effect, especially on the uterus, decreased considerably during the 10 days after the injections were discontinued.

There was definite co-operative activity with oestradiol dipropionate on the uterus and especially on the vagina, but not on the preputial glands (group VII).

*Histological changes* in the sex organs were similar in character but not in degree to those obtained with testosterone propionate [Korenchevsky & Hall,

1937]. With 150  $\mu$ g. a dioestrus uterus and metaplasia of the vaginal epithelium into small mucous cells were obtained, but with 1500  $\mu$ g. the progestational changes both in the uterus and vagina approached much more closely than with the propionate to those seen during pregnancy. Thus the dipropionate possesses, even more strongly than the propionate, this remarkable property of producing progestational changes in the sex organs of ovariectomized animals, without any previous sensitization with oestrogens. The histological changes produced by the injections were maintained during the 10 days' "rest" even less than were the weight changes: the uterus became atrophic, and the mucous metaplasia in the vagina partly, or in some rats entirely, disappeared.

*Co-operative and antagonistic activities of testosterone dipropionate and oestradiol dipropionate*

These two hormones when injected simultaneously show both co-operative and antagonistic activities.

Miescher *et al.* [1938] have shown that oestradiol dipropionate has a greater and more prolonged activity than pure oestradiol. This is also shown in the present experiments, when 6  $\mu$ g. of the dipropionate produced about the same effect by weight and histologically as 30  $\mu$ g. of pure oestradiol [Korenchevsky & Hall, 1937]. In the uterus, 6  $\mu$ g. of the dipropionate produced the same high cylindrical epithelium, transformed in places into squamous epithelium (metaplasia), as [Korenchevsky & Hall, 1938] that with 30  $\mu$ g. of oestradiol. This metaplastic epithelial change is important as indicating the first step in the "carcinogenic" activity of oestrogens.

Simultaneous injections of testosterone dipropionate and oestradiol dipropionate gave a picture typical of the female hormone. Thus oestradiol dipropionate is *antagonistic* to and overrules the activity of testosterone dipropionate. It is important that the doses used of testosterone dipropionate did not prevent the metaplastic changes produced by oestrogens in the uterus, while these changes were prevented by progesterone [Korenchevsky & Hall, 1938].

A *co-operative* activity of the dipropionates of testosterone and oestradiol was shown in a better recovery in weight and size of the uterus and vagina.

*Effect on female prostate and clitoris, and classification of the hormone.* The injections caused a great development of the female prostate and clitoris, similar to that described previously for the other male hormones [Korenchevsky, 1937, 2; Hall, 1938].

The female prostate, unlike the uterus and vagina, did not regress during the 10 days after the cessation of the injections (group IV). Moreover, 1500  $\mu$ g. and even 500  $\mu$ g. of testosterone dipropionate overstimulate the male organs, but 1500  $\mu$ g. are unable to restore the uterus to normal. These facts place testosterone dipropionate (using the classification suggested by Korenchevsky [1937, 1]) in the group of "bisexual hormones possessing chiefly male properties."

*Other organs, fat deposition and gain in body weight*

*Thymus and adrenals.* Testosterone dipropionate had a similar effect on the weights and histological structure of these organs in females as in males.

*Liver, kidneys, heart, fat deposition and gain in body weight.* The general effect on these organs appears to be similar in character to that in males, but weaker in degree and less constant. More numerous experiments, and especially of longer duration, are needed in order to come to a definite conclusion. No pathological changes were found histologically in the liver and kidneys.

The similarity of these changes in the two sexes is rather remarkable, as the effects of gonadectomy on most of the organs investigated were different in males from those in females. This will be discussed in more detail elsewhere.

When injected simultaneously (group VII) oestradiol dipropionate was found to neutralize more or less completely the activity of testosterone dipropionate on adrenals, hypophysis (increased weight), liver, heart, fat deposition and gain in body weight (decrease), while stimulation of kidneys was evident both in actual weight and per unit of body weight.

#### SUMMARY

1. In gonadectomized male and female rats the manifold effects of testosterone dipropionate are in general similar to those of testosterone propionate, with, however, some important differences.

##### *On male sex organs*

2. The effect of small doses of the dipropionate on these organs is weaker (e.g. on the prostate, by about 1.5 times) than that of similar doses of the propionate. With larger doses this difference is much less.

3. In contrast to the propionate, however, the effect of the dipropionate on the male sex organs is maintained (completely with 500  $\mu\text{g.}$ , partially with 150  $\mu\text{g.}$ ) for at least 10 days after the last injection.

##### *On female sex organs*

4. Testosterone dipropionate alone, without previous stimulation with oestrogens, can produce progestational changes in the uterus and vagina, much more similar to those found during pregnancy than the changes produced by the propionate.

5. After the cessation of the injections (small doses) the development of the vagina is maintained better than that of the uterus.

6. Testosterone dipropionate has very pronounced bisexual properties (including strong stimulation of the female prostate and clitoris), but, from a comparison of its "male" and "female" activities, it can be classified as a "bisexual hormone possessing chiefly male properties".

7. Testosterone dipropionate, in the dose used, did not prevent the metabolic changes produced in the uterine epithelium by oestradiol dipropionate.

##### *Non-sexual organs of both sexes*

8. Testosterone dipropionate influenced the thymus, adrenals, liver, kidneys, heart, fat deposition and gain in body weight in both sexes, but in most cases, with the doses used, the effects in females were less pronounced and less constant than in males.

9. Testosterone dipropionate and oestradiol dipropionate show both co-operative and antagonistic activities on sexual and non-sexual organs.

10. The experimental evidence obtained indicates a possible advantage of testosterone dipropionate over the propionate for clinical use.

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## VI. PREPARATION OF $\alpha$ -AMINO-ACIDS THROUGH $\alpha$ -OXIMINO-ESTERS

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AMINO-ACIDS free from biologically important impurities which may accompany those prepared from natural sources have been required in this department for the study of bacterial nutrition. Synthesis of simple amino-acids presents little difficulty by the usual routes, but serviceable methods are still required in certain cases, particularly if ring closure of intermediate derivatives is apt to occur. We have therefore extended to new compounds the ethyl acetoacetate synthesis of Bouveault & Locquin [1904; 1906] and Schmidt & Widman [1909] via  $\alpha$ -oximino-esters, since this method has also the merit of accessible starting materials. Difficulties in the earlier work have been minimized by using the convenient catalytic reduction of oximino-esters applied by Harington & Randall [1931] to hydroxyglutamic acid. This reduction may have considerable biological interest, since oximinosuccinic acid has been isolated by Virtanen [1938] as an intermediary in the production of aspartic acid during nitrogen assimilation by legume bacteria, of which process it probably constitutes the first stage.

Glutamic acid has been successfully synthesized in an overall yield of 39% as compared with 18.5% in the best method hitherto available from ethyl malonate [Dunn *et al.* 1931], and the even smaller yields from other sources [cf. Dunn *et al.* 1931]. Hydroxyproline has been prepared, but the yield was not superior to that of Leuchs [1905]. The synthesis of threonine has been attempted, but was discontinued following the publication by Adkins & Reeve [1938] of a satisfactory synthesis of that acid by an analogous method. Other preparations have been deferred in accordance with the needs of our work.

### EXPERIMENTAL

#### *Glutamic acid*

*Diethyl- $\alpha$ -acetylglutarate* was prepared by the following modification of the method of Clemo & Welch [1928]. To a cooled solution of sodium (4.03 g.; 0.175 mol.) in absolute alcohol (53 ml.) ethyl acetoacetate (44 ml.; 0.35 mol.) was gradually added, followed by ethyl  $\beta$ -chloropropionate (21 ml.; 0.175 mol.). The mixture was kept 3 hr. at room temperature and refluxed for 2 hr.; during the last  $\frac{1}{2}$  hr. the pH of an aqueous solution of a drop of the mixture remained at 9. Alcohol was removed at 100°, NaCl which separated during the process dissolved in the minimum quantity of water and the aqueous solution extracted with ether. The ethereal extracts were washed with very dilute HCl, dried with CaCl<sub>2</sub>, evaporated and the residue distilled. Excess ethyl acetoacetate distilled first, and the glutaric ester (31.8 g.; 79% theory) at 146–150°/12 mm.

*Diethyl- $\alpha$ -oximinoglutarate* was prepared from the acetyl compound according to Wislicenus & Grutzner [1909].

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*Glutamic acid.* The freshly prepared oximino-ester (16 g.; 0.075 mol.), platinum oxide (0.4 g.; Adams *et al.* [1932]) and anhydrous  $\text{Na}_2\text{SO}_4$  (15 g.) in acetic acid (60 ml.; redistilled) were shaken in hydrogen at a little over atmospheric pressure until the theoretical uptake had taken place (3 days). The solution was filtered, water added, the further precipitate of catalyst filtered off and the solution taken to dryness. The amino-ester remaining was immediately hydrolysed by refluxing with 5 *M* aqueous  $\text{HCl}$  (50 ml.), the product evaporated to 25 ml. under reduced pressure, cooled in ice and saturated with  $\text{HCl}$ ; the glutamic acid hydrochloride was collected after keeping for 12 hr. at  $0^\circ$ . Yield 9.0 g. A further crop of about 1 g. was obtained from mother liquors.

The salt was dissolved in water, freshly distilled aniline (20 g.) added, the solution warmed and ethyl alcohol added till crystallization began. The glutamic acid was crystallized from water and washed with alcohol; m.p.  $197^\circ$ . Yield 7.8 g., i.e. 39 % of the theoretical from the  $\beta$ -chloropropionic ester used. (Found: amino-N, 9.5 %;  $\text{C}_5\text{H}_9\text{O}_4\text{N}$  requires 9.5 %. *Hydrochloride*, m.p.  $198^\circ$ ; *picrolonate*: found, amino-N 3.4 %;  $\text{C}_5\text{H}_9\text{O}_4\text{N} \cdot \text{C}_{10}\text{H}_8\text{O}_5\text{N}_4$  requires 3.4 %.)

### *Hydroxyproline*

*$\alpha$ -Oximino- $\delta$ -chloro- $\gamma$ -valerolactone.*  $\alpha$ -Acetyl- $\delta$ -chloro- $\gamma$ -valerolactone [Leuchs, 1911]; B.P.  $135\text{--}137^\circ/1$  mm.) (17.6 g.; 0.1 mol.) was added with vigorous stirring to conc.  $\text{H}_2\text{SO}_4$  (20 ml.) cooled in ice-salt. Nitrosylsulphuric acid (12.7 g.; 0.1 mol.) in conc.  $\text{H}_2\text{SO}_4$  (12 ml.) was added during  $\frac{1}{2}$  hr., the whole poured on to crushed ice, the product extracted with ether, the ethereal solution washed quickly with water followed by aqueous  $\text{Na}_2\text{CO}_3$ , dried and evaporated. The oxime remained as an oil, decomposing violently at  $170^\circ$  on attempted distillation but separating from benzene as pale yellow crystals. Yield, 11 g. (67 %) m.p.  $118^\circ$ . (Found: N, 8.6 %;  $\text{C}_5\text{H}_6\text{O}_3\text{NCl}$  requires N, 8.6 %.)

*$\alpha$ -Amino- $\delta$ -chloro- $\gamma$ -valerolactone salts.* The oxime (8.2 g.; 0.05 mol.), platinum oxide (0.4 g.), anhydrous  $\text{Na}_2\text{SO}_4$  (5 g.) and glacial acetic acid (50 ml.) were shaken in hydrogen for 3 days; the solution was filtered and evaporated and the residue rubbed with alcohol. The amine acetate separated and was recrystallized from aqueous alcohol, yielding 2.9 g. (0.014 mol.) of colourless prisms m.p.  $177^\circ$ . (Found: amino-N, 7.0 %;  $\text{C}_5\text{H}_8\text{O}_2\text{NCl} \cdot \text{C}_2\text{H}_4\text{O}_2$  requires 6.7 %.) A similar yield of hydrochloride was obtained on reducing the oxime in alcoholic  $\text{HCl}$ .

*Hydroxyproline.*  $\alpha$ -Amino- $\delta$ -chloro- $\gamma$ -valerolactone acetate (4 g.) with saturated aqueous ammonia (80 ml.) was warmed at  $30^\circ$ . Titration of small portions indicated the halogen to be completely eliminated as  $\text{Cl}^-$  after 2 hr. Hydroxyprolines *a* and *b* were isolated according to Leuchs [1905]. Yields: *a*, 0.1 g., m.p. (d.)  $254^\circ$ . (Found: N, 10.4 %;  $\text{C}_5\text{H}_9\text{O}_3\text{N}$  requires 10.7 %); *b*, 0.7 g., m.p. (d.)  $244^\circ$ . (Found: N, 10.5 %.)

### *Threonine*

*Reduction of ethyl- $\alpha$ -oximinoacetoacetate.* Reduction and hydrolysis under the conditions used by Harington & Randall [1931] yielded only  $\alpha$ -aminobutyric acid; yield, 80 %. (Found: N, 13.4 %;  $\text{C}_4\text{H}_9\text{O}_2\text{N}$  requires 13.6 %.) It was not found possible to arrest the reduction at the hydroxy-acid stage.

*Ethyl- $\alpha$ -amino- $\beta$ -ketobutyrate hydrochloride.* This intermediary in the above reduction was isolated in order to attempt its reduction by other methods. Ethyl isonitrosoacetoacetate (8.0 g.) palladium charcoal (3.7 g.) and alcoholic  $\text{HCl}$  (50 ml. 2*N*) were shaken in hydrogen. Theoretical uptake occurred in 5 hr. The solution was filtered and evaporated under reduced pressure to a few ml., when the hydrochloride separated in white prisms. It was recrystallized from



alcohol with the addition of light petroleum. Yield, 62 %, m.p. 125°. (Found: N, 7.85 %;  $C_6H_{11}O_3N.HCl$  requires 7.7 %.) The salt exhibited the properties described by Gabriel & Posner [1894] and by Cherchez & Cherbuliez [1931] but was obtained in better yield and melted much higher than described by those authors. Attempted further reduction yielded only  $\alpha$ -aminobutyric acid or diethyl-2:5-dimethylpyrazine-3:6-dicarboxylate.

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## VII. ON THE RELATIONSHIP BETWEEN THE SULPHUR CONTENT AND THE ANTICOAGULANT ACTIVITY OF HEPARIN PREPARATIONS

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ABOUT 20 years elapsed between the discovery of heparin by Howell in 1916 and its chemical identification. Howell [1928] reported on preparations which must have been reasonably pure and showed a positive naphthoresorcinol reaction, indicating the presence of a hexuronic acid. Fischer & Schmitz [1933], as well as Charles & Scott [1933], also worked with preparations of a high degree of purity, considered by them to be crystalline or semi-crystalline. Fischer & Schmitz [1933] claimed that they had succeeded in preparing a crystalline brucine salt of heparin. The elementary composition of this heparin indicated that it contained neither sulphur nor nitrogen. Their statement was unjustified, however, for on ignition the samples yielded a small amount of ash and were thus not homogeneous but a mixture of brucine and alkali salts.

Heparin was found by Jorpes [1935] to be a polysulphuric ester of chondroitin or some closely related substance. When the amino-sugar was identified as glucosamine [Jorpes & Bergström, 1936] heparin seemed to be a mucoitin polysulphuric acid. The occurrence of sulphur, accidentally observed by Howell already in 1928, was later denied [Fischer & Lipmann, 1935; Schmitz, 1935], but sulphur was soon afterwards recognized as an essential component of the active substance [Charles & Scott, 1936].

A new contribution to the discussion was made by these latter authors, Charles & Scott, in that they claimed that they had succeeded in isolating a crystalline barium salt of heparin. This salt would thus have to be regarded as the first chemically pure preparation of the anticoagulant. The free acid, derived from the barium salt as well as from an amorphous benzidine salt, was believed to have the composition  $C_{25}H_{65}O_{50}N_2S_5$ , with 11.5% S. This statement has led to some confusion concerning the chemical nature of heparin, for all the evidence previously obtained indicated that heparin was not a definite chemical compound but a mixture of different polysulphuric esters, i.e. a polysaccharide (mucoitin) which could be esterified with sulphuric acid to a varying degree and might contain from 20 to 40% of sulphuric acid. With increasing S content there was a steady increase in the anticoagulant activity. The different esters, having different S contents, could easily be separated as brucine salts by fractionation in water. The organic skeleton appeared to be the same in all of them, hexuronic acid, hexosamine (glucosamine) and acetic acid together making up at least 90%. All this evidence was available in 1935.

It is quite natural that in the meantime those who have reviewed the subject have considered this alleged crystalline product to be pure heparin. They have not paid much attention to the fact that the amorphous preparations can contain 20% more S (up to 13.5% in the free acid) than the crystalline product, which has 11.5%. The more recent discussions have rather neglected the previously

established facts. Thus Charles & Scott [1936] did not feel convinced that heparin contained hexuronic acid and hexosamine, in spite of the fact that, by means of most reliable reactions for detecting sugars the Tollens-Lefèvre procedure and the pyrrole reaction with Ehrlich's reagent for amino-sugars, they had been shown to make up 90 % of the ash-free preparations, if acetic acid also is assumed to occur in heparin. They found a considerable part of the nitrogen to occur in  $\text{NH}_2$  groups. The amino group of heparin, however, is monoacetylated,  $-\text{NH}.\text{OC}.\text{CH}_3$ , as everywhere in the amino-sugars in nature. Of course part of it can become de-acetylated during the process of preparation, which includes heating with acids as well as with alkali, just as occurs in the preparation of chondrosine (mucosine) by acid hydrolysis. This negative aspect was most recently clearly expressed by Best [1938], who claims that "tests do not indicate the presence of glucuronic acid, which some previous observers had thought was present". The situation is somewhat perplexing when biochemists of to-day are unable to trace a substance which constitutes 25-30 % of the preparation and which was discovered by a physiologist 10 years ago. However, this matter need not be further discussed. The quantitative analysis according to Tollens-Lefèvre is quite reliable. The titration of the free acid obtained by electro-dialysis showed at an early stage that there was one carboxyl group to 2 or 3 sulphate groups. The titration curves also prove, as did the Van Slyke analysis, that the amino group is acetylated [Wilander, 1938].

In Charles & Scott's formula for the crystalline product there is one detail which immediately disproves its correctness. There are 2.5 atoms of S to each N atom. The same observation was made by Jorpes [1935] as the result of analysing the earliest samples of pure heparin available. It seemed improbable that heparin should contain 2.5 sulphate groups in each chondroitin (mucoitin) complex. A more plausible explanation was then obtained when it was found that the preparations could be divided by means of brucine into fractions having the composition of chondroitin (mucoitin) di- and tri-sulphuric acids and mixtures of these. With increasing S content there was, as mentioned, an increase in the anticoagulant activity. Additional analyses indicating the same thing were later published by Jorpes & Bergström [1937]. Heparin has therefore always been regarded by us as a mixture of mucoitin polysulphuric esters.

The question as to the homogeneity of the product of Charles & Scott, supposed to be crystalline, is consequently of great interest. It will also be of importance when the question arises as to what is to be an international standard of heparin. In order to check our results we have repeated our earlier experiments on a larger scale and have subjected the different fractions of heparin to a very careful S analysis and a thorough biological standardization. No corrections of our earlier views have been found necessary.

*The fractionation of ordinary pure heparin by means of  
brucine salts*

The heparin used was the commercial product supplied by Vitrum of Stockholm. It is a sodium salt prepared mainly in accordance with the Charles & Scott method and contains about 12 % moisture, 33 % ash, 2 % N and 9-10 % S. It is 8-10 times more active than the older commercial samples, its activity being fairly constant. This sodium salt is used in the intravenous treatment of thrombosis in man. The free acid was prepared by electro-dialysis against parchment in an apparatus constructed by E. Hammarsten [see Ågren, 1934]. Either 25 g. of heparin dissolved in 200 ml. were electro-dialysed for 48 hr. or smaller samples were treated for a shorter time. Electro-dialysis was continued until no

alkali was traceable with litmus in the cathode liquor. The acid was neutralized with brucine and the brucine salt fractionated in water. One part of it was soluble in water. In separating the insoluble fraction a clear mother liquor was obtained only after repeated freezing and thawing. This fraction was dissolved in boiling water and again frozen out. By repeating this procedure a series of mother liquors was obtained and a final less soluble brucine salt. It was also possible to separate a small fraction which was insoluble even in a large volume of boiling water. This last fraction showed the highest S content and the greatest anticoagulant activity.

The various brucine salts were treated with a small excess of NaOH and the brucine was removed with chloroform. The solution was then neutralized with HCl against litmus and the heparin precipitated with 1.5 vol. acetone. The precipitate was collected the following day, dissolved in a small amount of water and reprecipitated with alcohol.

For S analysis the substance was fused with carbonate-nitrate in a porcelain crucible. The precipitate of BaSO<sub>4</sub> was collected on a Neubauer-Gooch platinum-iridium filter, ignited, washed with HCl and dried. The error of the method is insignificant. On two occasions samples were sent to Dr Schoeller of Berlin for control analysis, the same figures then being obtained as by the ordinary method.

The biological standardization was performed on fresh ox blood as described by Jorpes [1935]. This method easily discloses differences of 20 % and usually differences as small as 10 % [see also Wilander, 1938]. At least 8 stands with 10 test tubes (5 standard and 5 unknown) were used for each standardization, for some of them 14 or 16 stands. The figures in Tables I-III ought to be correct to within 10-15 %.

Two preparations of 25 g. each were fractionated; the results are presented in Tables I and II. The experiment given in Table III is incomplete.

Table I. *Fractionation of 25 g. heparin as brucine salts. Yield of sodium salts after removal of the brucine*

Source of substance	Wt. air-dry substance g.	Anticoagulant activity % standard	S % of dry substance
1st mother liquor	3.37	60	7.42
2nd mother liquor	5.30	45-50	7.01
3rd mother liquor	5.25	100	10.97
Less soluble brucine salt	2.30	125	11.74
Insoluble brucine salt I	0.266	150-160	11.88
Insoluble brucine salt II	0.238	150	11.96
	<u>16.72</u>		

Table II. *Fractionation of 25 g. heparin of standard potency as brucine salts. Yield of sodium salts after removal of the brucine*

Source of substance	Wt. air-dry substance g.	Anticoagulant activity % standard	S % of dry substance
1st mother liquor	5.12	20-25	8.52
2nd mother liquor	6.52	75-80	9.58
3rd mother liquor	2.73	100-110	11.58
4th mother liquor	1.51	100	11.20
Less soluble brucine salt	0.61	140-150	12.13
Insoluble brucine salt I	0.32	130	11.72
Insoluble brucine salt II	0.09	150-160	12.26
	<u>16.90</u>		

Table III. *Fractionation of standard heparin as brucine salts. Yield of sodium salts from the most soluble fractions*

Source of substance	Anticoagulant activity % of standard	S % of dry substance
1st mother liquor	75	9.31
2nd mother liquor	80	10.41

Table IV. *Sulphur contents and anticoagulant activities of different heparin preparations*

S % of dry substance	Anticoagulant activity % of standard
7.01	45-50
7.42	60
8.52	20-25
9.31	80
9.58	75-80
10.41	80
10.97	100
11.20	100
11.58	100-110
11.74	125
11.72	130
11.88	150-160
11.96	150
12.13	140-150
12.26	150-160

It is evident from Table IV, where the samples are arranged in a series, that the anticoagulant activity increases with increasing S content. Only one sample (no. 3) out of 15 breaks the series, and that was obtained from the first mother liquor of this preparation. It is also apparent from the table that further additions of S cause a greater increase in the activity if introduced in the highly esterified samples, thus indicating that it is the abundance of ionic charges that makes the polysaccharide an anticoagulant. The bulk of evidence now available indicates that the activity is due to the ionic charge of heparin [Jorpès, 1938].

The question arises whether the preparations with lower S content consist of highly esterified heparin and impurities. It has already been shown that their organic skeleton is the same, i.e. mucoitin. The content of uronic acid is considerably higher in these samples than in those with high S and ash contents. The amino-sugar is also demonstrated to be the same, glucosamine. The original assumption that ordinary heparin is a mixture of different poly- (di- and tri-) sulphuric esters thus seems to be justified.

The properties of the heparin preparations are strongly influenced by the introduction of additional sulphuric acid groups. This makes their barium and brucine salts less soluble or insoluble in water. The salts of the samples with 7.0-7.42 % S behave like salts of chondroitin sulphuric acid, and they are not precipitated with barium chloride or barium hydroxide. On the other hand, the fractions with a content of S greater than 10.41 % give less soluble or insoluble barium and brucine salts. This excludes the possibility that the former fractions contain considerable amounts of the highly esterified stronger heparin. In fact, an attempt was made to fractionate one of these samples (7.01 % S) once more with brucine. After electro dialysis the solution was neutralized with

brucine. No insoluble brucine salt was precipitated. After cooling the concentrated solution, part of the brucine salt separated out and was removed in the centrifuge. From 5 g. containing 7.01 % S there were obtained 0.97 g. of a fraction containing 5.17 % S and 2.15 g. of a fraction containing 7.80 % S. The anticoagulant activities were 15 and 50 % of standard respectively. As could be expected, at least the first fractions shown in Table IV are not homogeneous, but they do not contain any considerable amount of highly esterified heparin.

#### *Standard heparin*

It will become necessary in the future to have a reference substance, a standard of heparin, as is already the case with other biological products used in medicine. A homogeneous crystalline product, if available, would be valuable for that purpose. However, the amorphous preparations of the pure liver heparin already have a very constant strength. For this reason Bergström *et al.* [1937] suggested that these should be used as standard. The same constant potency was found by Murray & Best [1938]. The potency of these liver preparations is sufficiently constant for practical use, varying by not more than 10–15 % in different preparations. This strength has been used by us as standard. We express the anticoagulant activity in mg. of this standard, considering this to be the most practical expression for that activity.

Recently at least six different units have been suggested by various workers, a tendency which would appear undesirable. Any method of assay may of course be applied in order to check the potency of a preparation against a standard, but the strength can hardly be expressed in a simpler way than in milligrammes of this standard. There is no reason for the use of smaller units, for in blood analysis as well as in therapy quantities of the order of milligrammes will be dealt with.

#### CONCLUSIONS

It is evident from Tables I–III that the anticoagulant activities of different heparin preparations obtained in a similar manner from the liver, vary with their S contents. Preparations of liver heparin containing from 7 to 12.26 % S show potencies varying from 45 to 160 % of the activity of the standard heparin from which they are derived. Pure liver heparin is a mixture of mucoitin polysulphuric esters which can be separated from each other as brucine salts. The starting material has a fairly constant strength and is used as heparin standard, the anticoagulant activity being expressed in milligrammes of this standard.

These preparations are sodium salts of heparin. The most potent samples, which contain about 10 % Na, contain about 13.5 % S in the free acid. For this and other reasons the alleged crystalline barium salt of heparin isolated by Charles & Scott in 1936, which has a content of S corresponding to about 11.5 % in the free acid, cannot be considered as the anticoagulant substance itself. Its homogeneous nature must be doubted. In any case, when this substance is discussed it must be kept in mind that it is merely a fraction taken out from the series of different polysulphuric esters of mucoitin which constitute heparin.

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## VIII. A NEW TYPE OF MICRORESPIROMETER

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### INTRODUCTION

MANOMETRIC methods are among the most useful and widely applied techniques in the field of biochemistry. The respirometers of Barcroft or Warburg are most commonly used, and the theory and practical manipulation of these and other types has been summarized by Dixon [1934].

These instruments are capable of measuring gas exchanges of not less than about 30  $\mu$ l./hr., so that 5–50 mg. of tissue are normally used. However, there is a need for methods of measuring very much smaller gas changes, and several instruments capable of measuring O<sub>2</sub> uptakes of 1  $\mu$ l./hr. or less have been described during the last decade. Stefaneli [1937] has described a miniature Barcroft-Warburg type of instrument. In others the respiration chamber is connected to a horizontal length of capillary tubing, the movements of a drop of fluid in the latter indicating the changes in volume [Fenn, 1927; 1928; Jackson *et al.* 1930; Schmitt, 1932; 1936; Gerard & Hartline, 1933; Victor, 1935; Waddington *et al.* 1936 and Duryee, 1936]. An entirely different method described by Linderström-Lang [1937] and extended by Needham & Boell [1939], is based on the principle of the Cartesian Diver.

While each of these types possesses certain advantages, it appears that none of them in their present form can carry out on a micro-scale all the kinds of measurement which can be done on a larger scale in the Barcroft or Warburg apparatus.

The apparatus described below employs a principle which has so far not been used in a respirometer. As regards sensitivity, it is capable of measuring uptakes and outputs of about 1  $\mu$ l./hr. with an accuracy at least as great as that of the Warburg apparatus; it is therefore less sensitive than some of the instruments described above. However, unlike these others, it possesses all the potentialities of the Warburg apparatus. It can be assembled at one temperature and used at a widely different one; it can be filled with any gas mixture, and two or more separate fluids contained in it can be mixed at any desired time during an experiment. The fact that it can be used under sterile conditions is an additional advantage.

### PRINCIPLE

The respiring material rests in a completely closed chamber, one wall of which consists of a thin sheet of mica. Two plane mirrors are attached to the latter, and when the volume of the gas in the chamber changes, the mica will bulge (either inwards or outwards) with the result that the mirrors will tilt in opposite directions. By applying a positive or negative pressure to the *outside* of the mica membrane, the latter may be brought back to its original position, a simple optical system being used to detect when the mirrors are in the same plane. Then, knowing the volume of the gas space and the change in pressure in it, the amount of gas absorbed or given out may be calculated.

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## CONSTRUCTION

The component parts of the apparatus may be described under five headings:

- (1) The chamber containing the tissue.
- (2) The metal case containing the chamber.
- (3) The optical system.
- (4) Thermostat, with heaters, thermoregulator etc.
- (5) The pressure-regulating mechanism and manometer, and arrangements for filling the chambers with any desired gas mixture.

(1) *The respiration chamber.* This consists of two plane slabs of glass 25 mm. square, one 3–4 mm. thick, and the other about half this thickness. The thicker of the two—which will be referred to in the rest of this paper as the “cup”—has a cavity of 40 to 80  $\mu$ l. capacity in one side, in which lies the material being studied. The inside of the cavity, which may be of any shape, is lined with wax, so that two or more separate drops of fluid may be placed in the different arms of the cavity without mixing. (Fig. 1*a* shows a “cup” capable of holding

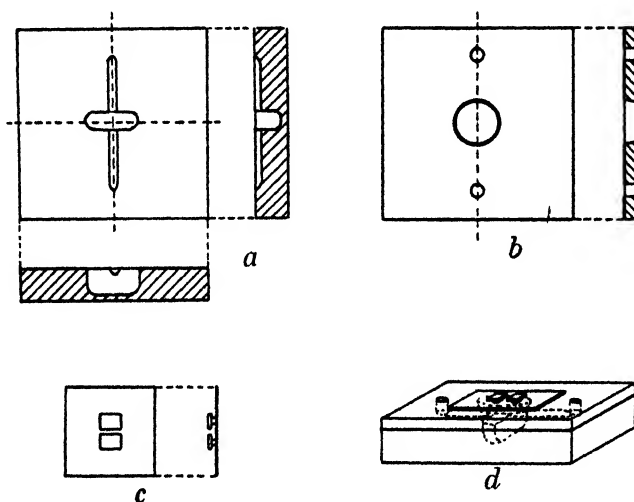


Fig. 1. Components of respiration chamber. *a*, “cup”. *b*, “plate”. *c*, mica membrane with mirrors attached. *d*, complete assembly.

two separate drops of fluid; Plate 1*A* shows “cups” for two, three and four drops of fluid.) The “cup” also has two grooves cut in the plane surface, which lead to the cavity, as shown in Fig. 1*a*.

The thinner slab—referred to in this paper as the “plate”—has a central hole about 7 mm. in diameter, which is covered on one side by the mica membrane. The “plate” (Fig. 1*b*) also has two smaller holes, 18 mm. between centres, which coincide with the ends of the grooves in the “cup” when the two are in apposition. Fig. 1*d* shows a complete assembly of “cup”, “plate” and mica ready to be placed in the brass case.

The respiration chamber may be filled with any desired gas mixture by leading the latter through one hole in the “plate”. (The way in which this is done will be understood after reading the following section.) The gas enters the respiration chamber through one groove and leaves it by the other, and after a sufficient stream of gas has been passed, the chamber can be completely closed by rotating the “cup” relative to the “plate”.

The "cup" cavities can be made by drilling a number of holes in the glass slab with a china-riveter's diamond drill [Heatley, 1938], and then enlarging them and running them together by means of a suitably shaped carborundum dental burr; the holes in the "plates" are made with the same tools. Up to the present the "plates" have been made from microscope slides carefully selected for flatness of surface; optically worked glass has been used for the "cups",<sup>1</sup> but good quality plate glass can also be used.

The mica for the membrane should not be thicker than  $18\ \mu$ , preliminary tests having shown that even very much thinner sheets are apparently quite impervious to gases. A freshly split mica sheet of the required thickness is divided into squares of 12 mm. side, by cutting it between two sheets of paper—preferably squared paper—with scissors. The mirrors are made from no. 3 cover-glasses, which are silvered, or better, aluminized, and cut up into rectangular pieces measuring  $2 \times 3$  mm. These are stuck to small pieces of cover-glass 1 mm. square, which in turn are fixed to the mica (Fig. 1c); the insertion of the glass stalk makes the component less fragile and more sensitive. It is a considerable help when cutting the cover-glasses with a diamond chip, to lay them on a piece of oiled silk on plate glass. Bakelite varnish no. V-5209/2 has been used for attaching the mirrors to the mica, and though they easily become detached, a more suitable adhesive has not yet been found. (Cements which cannot be autoclaved, or which are affected by water, have not been tried.)

(2) *The metal case.* Considerable latitude is permissible in the design of this component, and the drawings which are appended are of the cases which have actually been used by the authors.

Fig. 2 is self-explanatory. The main body of the case is built of the thick brass disk *A*, to the back of which a thin disk *B*, having a central square hole  $25.5 \times 25.5$  mm., is permanently fixed by two countersunk screws. A detachable disk *C*, having a central hole about 22 mm. in diameter, has three curved slots through which the three threaded rods *D* on the main body of the apparatus will pass. Projecting from one surface of *C* are eight pins enclosing an imaginary square of 25.5 mm. side. *A* has a central hole *E*, 7 mm. in diameter, which is closed on the outside by a glass window. The brass rod *J* acts as a handle, and by means of a hook soldered to it the case can be suspended from a horizontal bar fixed across the thermostat.

The "cup", "plate" and mica having been assembled into a unit (Fig. 1d), are placed mica downwards on to the main body of the case, which is held horizontally as in Plate Ia, *b*, so that the mirrors lie in the central hole *E*, facing the glass window. The rotating back *C* is now placed on top of the glass parts with the eight pins surrounding the "cup", and the plain and spring washers are slipped on to the rods *D*, followed by the nuts *F*, which are tightened. It will now be seen that with the help of the two partially sunk rubber washers *I*, the two outer holes in the glass plate are making gastight contact with the tubes *G* and *G'* of the case, enabling gas to be passed through the respiration chamber. Fig. 3 shows the components of the whole assembly in an expanded diagrammatic form.

The slots in the disk *C* allow it to be rotated through an angle of  $60^\circ$ , and the pins projecting from it will force the cup to rotate with it. On the other hand the "plate" is partially sunk into the square hole in *B* and so will be held stationary. Thus the rotation of the "cup" relative to the "plate", which is required to close the respiration chamber, is carried out with ease

<sup>1</sup> Blanks of optically worked glass suitable for the "cups" may be obtained from Henry Hughes and Son, Ltd., Husun Works, New North Road, Barking, Essex.

and accuracy. Plate IA shows a photograph of some of the components already mentioned.

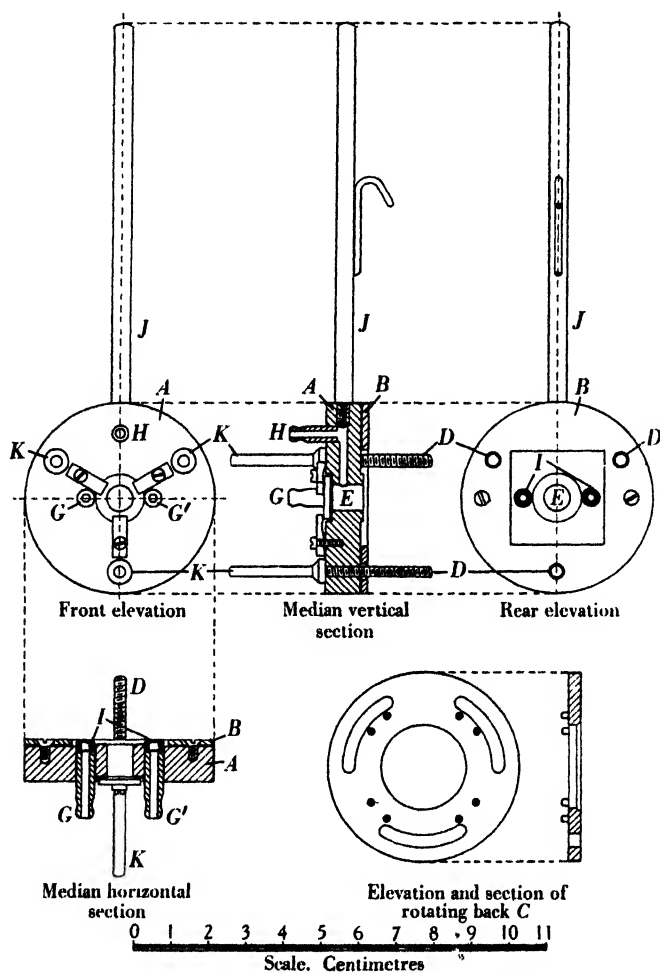


Fig. 2. Diagram showing construction of brass case.

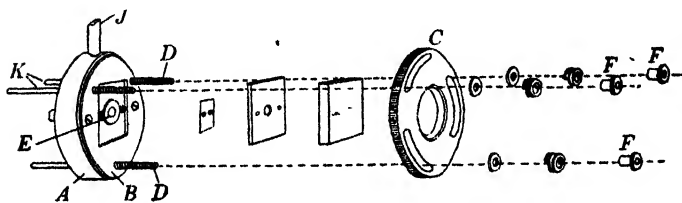


Fig. 3. Diagram showing disposition of components of brass case and respiration chamber.

In Fig. 2 will be seen a third tube, *H*, which leads directly into the space *E* between the glass window and the mica membrane; this tube is attached to the manometer and pressure-regulating apparatus, by which any bulging of the

mica can be corrected. (The bulging is detected by the optical system described in the next section.)

Some simple method for rapidly and accurately aligning the metal case with the optical system (when readings are to be taken) is required. This is done by placing the legs *K* of the case against a glass observation window let into the side of the thermostat, and preventing the case from rotating or sliding in a plane parallel with the window by a fixed V-shaped plate; all that is required now is some means for pressing the case against the window, and one simple mechanism for doing this is shown in plan and elevation in Fig. 4. The observation window is a small yacht decklight which is fixed to the thermostat wall by five bolts, three of which project inside and are used to secure the brass aligning-plate *L*. This has a V-shaped cutaway portion in which rest the legs *K* of the case. A stout pillar *M*, attached to this plate, is linked by the two freely pivoted strips *N* and *N'* to the rod *O*. An extension spring of stainless steel, anchored to suitable positions on the strips *N* and *N'*, ensures that *O* will press firmly against the back of the respirometer case. *O* is conveniently controlled by a knob (above the level of the water in the thermostat) which is rigidly connected to *N* (or *N'*) by the rod *P*. (In Fig. 4 the decklight, is shown in section in the plan and in dotted outline in the elevation.)

(3) *The optical system.* This is shown in its simplest form in Fig. 5a. An achromatic converging lens *V* is placed in front of an illuminated slit *S* so that an image of the latter would be formed, say, 70 cm. behind the lens. The mirrors *W* of the mica membrane are placed in the path of the convergent rays, some of which are reflected back to form an image on a ground glass screen *X*. When the mirrors are in parallel planes a single image of the slit will be seen, but as soon as the mica bulges one way or the other, the mirrors will tilt in opposite directions and the single image will split into two.

Even when special care is taken in the mounting of the mirrors on the mica, it will be found that only in a few cases are the planes of the mirrors approxi-

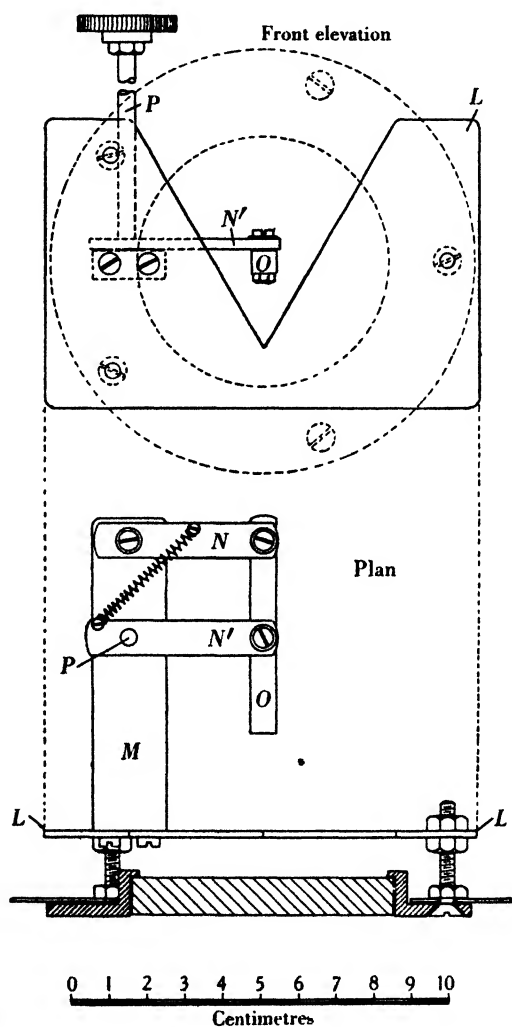


Fig. 4. Mechanism for holding brass case.

mately parallel; in the remaining cases the two images of the slit will be wide apart, and the zero position (i.e. when the two images fuse into one) can only be obtained when the mica is made to bulge. It is obvious that the membrane is most sensitive to deformation when it is flat, the sensitivity falling off rapidly as the bulging becomes greater. To avoid this loss of sensitivity a compensating arrangement of mirrors is provided by which the two images may be made to coincide, even when the pencils of rays from the two mica mirrors are widely

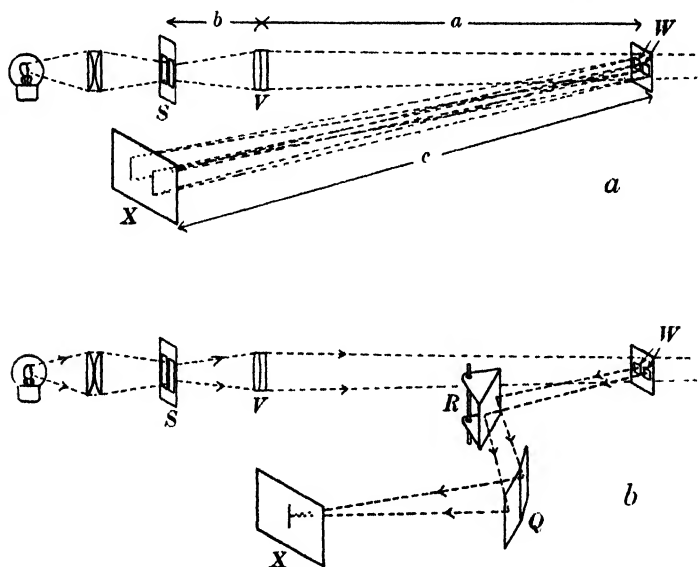


Fig. 5. Optical systems: *a*, in simplest form; *b*, with divided mirror.

divergent. Fig. 5*b* shows the principle of the method employed. Before falling on the ground glass screen, both pencils of rays are reflected from the vertically divided mirror *Q*, one half of which can be rotated about the dividing line. The tilt of the mirrors attached to the mica may be such that both pencils of reflecting rays, although diverging, may fall on the same half of the divided mirror *Q*. This contingency is met by interposing between the mica and *Q* another rotating mirror *R*, which can be so adjusted that one ray will fall on each half of *Q*.

When initially divergent rays are made to form a single image by the use of the divided mirror *Q*, there will be two different pressures which can give a zero reading, according to whether the rays are crossed before impinging on *Q* or not. The correct reading is easily selected, but this point may cause confusion at first. (At the correct reading a small change in pressure will cause the images to move a considerable distance relative to each other—at the other null point the same pressure change will cause only a small displacement of the images.)

The illuminated slit was found to be more efficient than a straight filament lamp.

The relation of the components of the optical system must be a matter of compromise. The distance *c* (Fig. 5*a*) must be great in order to obtain an efficient optical lever; but if it is made too big relative to *a* the area of the mirrors *W* will be small at that position in relation to the cross-sectional area of the converging beam, and much light will be lost. If, however, *b* is large, the lens will collect only a small amount of light from the slit; but if *b* is too small in

relation to  $a+c$ , the magnification of the slit will be excessive and loss of definition and brilliancy of the ground glass image will be the result.

Some light will be reflected from the window of the brass case, causing a secondary image, and another image may be formed in the same way from the thermostat window. The first unwanted image may be eliminated by having the small window in the case bedded down on a washer of uneven thickness, so that it is no longer perpendicular to the optical axis. The second may be eliminated by inclining the case at, say,  $5^\circ$  to the vertical, either by slightly lengthening the bottom leg  $K$ , or preferably by a suitable packing, such as a microscope slide, between it and the thermostat window. The optical system must be tilted a corresponding amount so that its optical axis is still perpendicular to the two mica mirrors  $W$ . The thermostat window will now no longer be perpendicular to the optical axis, and any reflexions from it will not interfere.

The exact arrangement of the optical system will depend on the characteristics of the individual components, but Fig. 6 shows a horizontal section through the optical box of the instrument at present in use. An image of the filament of the 36 W. 12 V. car headlamp bulb  $U$  is focussed on the vertical slit  $S$  by means of the condenser  $T$ . The achromatic lens  $V$  has an approximate focal length of 9 cm., and throws a beam of light from the slit on to the mirror  $R$ . This rotates about a vertical axis and is controlled by the screw  $Y$ , backlash being prevented by a spring. The beam of light passes through the thermostat window (shown in section in Fig. 6) and falls on to the two mirrors mounted on the mica membrane, from which two pencils of light are reflected back on to  $R$  and thence to the divided mirror  $Q$ . When the pencils of rays are initially divergent,  $Y$  may be adjusted so that one pencil of rays falls on to each half of the mirror  $Q$ : then by tilting one half of the latter by rotating the graduated drum  $Z$  (which is fixed to a threaded rod) the two separate images of the slit may be made to superimpose. Mirror  $X$  is merely for the sake of convenience, as it allows the ground glass screen to be at the front, instead of at the back of the apparatus; the screen is situated above the drum  $Z$ , and therefore is not shown in Fig. 6. In Plate I b the hood which screens it from stray light while it is being viewed can be seen. A ball bearing hinge such as that described by Baker [1938] is used for the divided mirror  $Q$ .

(4) *Thermostat, etc.* The tank at present in use is made of copper and measures 65 cm. long by 28 cm. broad by 25 cm. high. It is heated electrically by three immersion heaters of 250 W. each, and one kettle unit taking either 100 or 700 W. The thermoregulator is of the ordinary toluene-mercury type, the toluene-containing part being thin-walled glass tubing about 1 cm. in diameter. Part of the heating current is controlled by the thermoregulator, and part is fed continuously: the advantage of this arrangement—which is well known, but not used as often as it might be—is that heat is continuously supplied to the bath at a rate which would allow it to cool only very slowly; hence the intermittent heating current need only be small, and fluctuations in temperature caused by thermoregulator lag, etc., will be correspondingly reduced. The thermostat can be warmed from room temperature to  $37^\circ$  in about an hour by employing the 700 W. heater and one of the 250 W. ones. When the bath has warmed up, the heaters are connected as follows:

*Intermittent* (controlled by relay): 700 W. heater in series with one 250 W. heater.

*Continuous*: two 250 W. heaters in series.

In a very warm or very cold room another of the many possible heater combinations may be more suitable.

The thermostat is stirred by a three-bladed vane driven by an electric gramophone motor situated under the tank. The stirring is efficient, noiseless and trouble-free, and by having the motor under the tank (private communication from Prof. R. A. Peters) more room is left at the top, and troublesome pulleys are avoided.

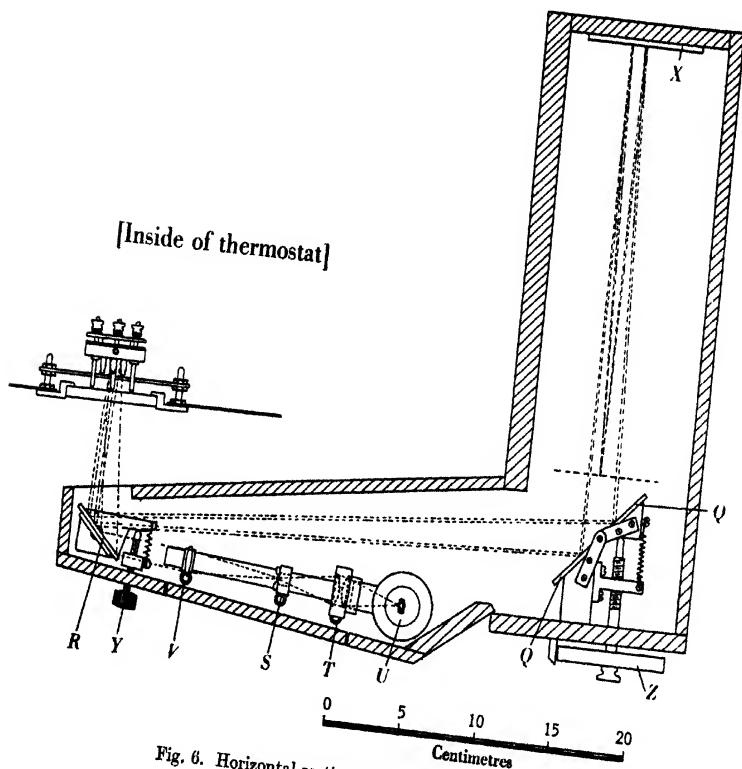


Fig. 6. Horizontal section through optical box.

A transformer giving an output of 3 amp. at 14 V. is housed behind the control panel (Plate 1B). This feeds the 36 W. car headlamp bulb used in the optical system, and also a low tension metal rectifier which delivers 2 amp. at 9 V.: a three-way switch enables either of these circuits, but never both, to be connected, whilst the third position of the switch is "off". The current from the rectifier activates the electromagnet by which two drops in the respiration chamber may be mixed (for details of this see below). An intermediate transformer tapping at 4 V. supplies current for working the thermoregulator relay, which is also situated behind the control panel.

(5) *The manometer and pressure-regulating apparatus, etc.* The principle of this is shown in diagrammatic form in Fig. 7. In the actual instrument the two reservoirs, consisting of 3 oz. bottles suspended horizontally, are attached to opposite sides of an endless chain passing round a system of pulleys and sprockets. A control wheel fixed to one of the latter enables the pressure in the system to

be varied (see Plate I B and Fig. 7). Those outlet tubes which are not connected to tube *H* of the brass cases (see Fig. 2), are closed by rubber caps. It is convenient to read the level of fluid in both arms of the manometer, thus avoiding errors through drainage; the manometer scale should be at least 60 cm. long if the instrument is ever to be used for respiratory quotient determinations (to be described in a subsequent paper).

For filling the cups with any desired gas mixture, a main tube having six side tubes is connected to the gas source; a separate bubbler, containing water and immersed in the thermostat, is interposed between this manifold and each respiration vessel, partly to moisten the gas, and partly to enable the rate of passage of gas in each individual case to be checked. It is important that the gas should be as nearly saturated as possible before entering the vessel, as otherwise evaporation of the droplets may occur, with alteration in the volume of the fluid and thus of the vessel constants. It was found that when dry  $O_2$  was passed through the respiration chambers via the bubblers, for 10 min. at  $37^\circ$  at the rate of 5–10 ml./min., only about 0.5  $\mu$ l. of fluid was lost from a drop of 12  $\mu$ l. of distilled water. However, though this would cause a negligible error in the vessel constants, it might have a deleterious effect on delicate cells by making the medium hypertonic. The evaporation can be reduced still further by inserting a plug of damp cotton wool in the rubber tube connecting the case to the bubbler.

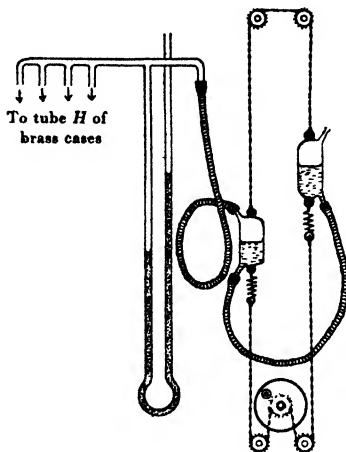


Fig. 7. Diagram showing principle of manometer and pressure regulator.

A thermobarometer is required, as in the Warburg technique, to compensate for any changes in thermostat temperature or barometric pressure. Since the volume of the vessel used as thermobarometer is immaterial, a simple bulb connected to a manometer of capillary tubing serves the purpose. Apparently no error is introduced by the difference in shape or size of this vessel compared with the respiration chambers, though should temperature regulation not be good, errors would undoubtedly arise.

#### CALIBRATION AND PROCEDURE

**Grease.** The respiration chamber is made airtight by films of grease between the component parts. Many different kinds of grease were examined and found to be unsuitable on account of their physical or chemical properties (consistency, absorption of gases etc.). For working at  $37^\circ$  B.D.H. winter grade rubber grease was found to be entirely satisfactory. The exact method of applying the grease is important, and too much or too little may spoil the experiment. It is most suitably applied with the ball of the finger, being patted on, rather than spread or smeared. Of the surface to which the mica is to be fixed, only a small central area need be greased, and before the mica is placed in position the edge of the central hole should be wiped free from excess grease, and this is most easily done with the ball of the clean little finger; if this is omitted, a ridge of grease may be squeezed into the cavity which will prevent the mica from moving perfectly freely.



*Effect of concentration of alkali, etc.* In all microrespirometers where the gas volume is small, and the surface/volume ratio of the fluids is great, isothermal distillation from one drop of fluid to another may occur; if the different drops have different initial osmotic pressures, they will tend during the course of the experiment, to attain the same osmotic pressure, with resulting changes in the concentration of the solutes in each drop. This can easily be demonstrated by actual measurement of the volumes of the drops before and after the experiment, and at 37° changes of this kind may occur in a relatively short time.

An important example of this effect is seen when strong alkali is used to absorb CO<sub>2</sub>. This is illustrated by the results of an experiment in which the O<sub>2</sub> uptake of a suspension of lymphocytes was measured in the presence of (a) 1% and (b) 10% NaOH. The actual values obtained were

(a) In 1% NaOH: 0.25, 0.21, 0.23  $\mu$ l. O<sub>2</sub>/hr.

(b) In 10% NaOH: 0.14, 0.13, 0.19  $\mu$ l. O<sub>2</sub>/hr.

It will be seen that the rate of O<sub>2</sub> uptake is markedly lower in the latter case. It seems that lymphocytes are considerably more sensitive to osmotic changes than other tissues, but in any experiment where concentration of medium is to be avoided, strongly hypertonic solutions of alkali, etc., should not be used.

This is a point which has sometimes been overlooked in previous work with microrespirometers, and the results obtained may therefore, in some cases, be erroneous.

*Method of mixing drops.* One or more of the droplets inside the respiratory chamber may be mixed by dragging a small iron ball from one to the other by means of an electromagnet. Mr Leemans has invented a method of applying a thin coating of glass to small balls of a special magnetic alloy ("platinersatzdraht")<sup>1</sup> but an alternative method of protecting the mixing ball from corrosion is as follows. Steel bearing balls,  $\frac{1}{16}$  in. in diameter, are given several coats of Bakelite varnish no. V-5209/2, each coat being polymerized by stoving before applying the next. The balls are then heated to 100° in paraffin wax (M.P. 50°) for some minutes, the surplus wax being removed by rolling the balls on hot filter paper; by this means any flaws in the Bakelite coating are blocked with wax. They are then rolled in the palm of a warm, but clean and dry hand with some well washed kaolin; this is necessary because the paraffin-coated balls are otherwise not readily wetted, and hence are inefficient mixers. As Bakelite is attacked by certain reagents (e.g. KMnO<sub>4</sub>), balls prepared by this second method cannot be used in the presence of such substances. Other ways of protecting steel balls from corrosion, such as gold or nickel plating, have been tested and found unreliable.

*Cleaning and preparation of the apparatus.* At the conclusion of an experiment the mica membrane is dragged off the "plate" and placed on a piece of clean filter paper. The adherent grease is removed from the upper surface by means of a blunt scalpel, and from the under side by moving the mica over the surface of the filter paper. The "plate" and "cup" are then cleaned in hot chromic acid, or better, in sulphuric-nitric acid mixture. They are rinsed under the tap, then in distilled water, and dried in the oven. The "cup" cavities are then lined with paraffin wax (M.P. 70°) by carefully heating them over a small flame and touching minute shavings of wax on to the hot surface; before the "cup" has cooled completely it is rubbed face downwards on a piece of clean silk in order to remove any wax which may have spread from the cavity to the plane surface.

<sup>1</sup> Mr Leemans, who is glassblower at the Clarendon Physics Laboratory, University of Oxford, is prepared to supply these balls.

In preparing for an experiment the "plates" are greased in the manner described above, and the mica squares carrying the mirrors are placed in position on them. The greased "plates" (with mica membrane attached) are best kept on a special stand protected from dust until they are required. As soon as each cup is charged with the material being studied, it is covered with a plate. The flat raised rim round the central hole *E* (Fig. 2) in a brass case is carefully wiped and greased, the inner edge being wiped free from excess grease by the ball of the little finger. The "cup"-plate-mica assembly is placed in position, the rotating back is added, followed by the plain and spring washers and the knurled nuts which are screwed up evenly all round. At this stage the numbers of the "cup" and "plate" are noted; to reduce the possibility of mistakes, the "cups" are numbered in Roman, and the "plates" in Arabic, characters. Each knurled nut is unscrewed one half turn to allow the back of the case to be rotated more easily, and the whole is placed in the thermostat and connected to the manifold leading to the manometer, and also to one of the bubblers. At some time during the passage of the gas, the case is clamped against the thermostat window and the zero point is examined. If the two images of the slit on the ground glass screen can be made to coincide by a pressure of not more than, say,  $\pm 30$  mm. of water, nothing further is done; but if a bigger pressure is required (i.e. if the mirrors on the mica are far from parallel), the pressure is kept at zero and the two images are made to coincide by adjusting the mirror *Q* by means of the calibrated drum *Z*. The drum reading is noted, and whenever a reading is taken of that particular respiration chamber, the drum is always set to the same reading.

When all the zero readings have been taken, the gas stream is stopped and the vessels are closed by rotating the back of each case backwards and forwards five or six times; after 2 or 3 min. temperature equilibration is attained and readings may be commenced. Each case has three rubber tubes leading from it, and when several cases are being used it is convenient to keep these tubes out of the way by suspending them in hooks attached to elastic cords which dangle from a permanent framework. The latter and the elastic and hooks can be seen in Plate I B.

*Determination of constants.* The constants of the respiration chambers are determined as for the Warburg vessels [*vide* Dixon, 1934]. The constant, *K*, is therefore given by the formula

$$K = \frac{v_G \frac{273}{t} + v_F \alpha}{P_0},$$

where  $v_G$  = volume of gas space,

$v_F$  = volume of fluid,

$\alpha$  = solubility coefficient of the gas in the liquid concerned,

$t$  = the absolute temperature,

$P_0$  = the normal pressure (760 mm. Hg) in mm. of manometric fluid. If

$D$  is the density of the latter, then  $P_0 = \frac{760 \times 13.6}{D}$ .

Then, where  $h$  is the manometric reading observed, the amount of gas,  $x$ , evolved or absorbed =  $hK$ .

The volume of fluid,  $v_F$ , can be measured by a pipette, or by determining the increase in weight of the tared "cup" after adding the fluid.

The total volumes of the "cups" and "plates" are determined initially by weighing the amount of mercury required to fill completely the central space. In the case of a "cup" this is done by filling with mercury, laying a plane slab of glass on top, and squeezing out the excess of mercury. Adhering globules

of the latter are brushed off, and the mercury is transferred to a tared watch-glass, which is reweighed. The volume of the central cavity of a "plate" is found in the same way, except that the "plate" is squeezed between two slabs of plane glass. The volume of the gas space,  $v_G$ , is obtained by subtracting the volume of fluid,  $v_F$ , plus the volume of the mixing ball (if used) from the total volume.

A number of factors suspected of altering the effective volume of the whole assembly were investigated, since any alteration in the volume of the vessel will introduce corresponding errors in the constant. The first of these factors was the thickness of the film of grease between the components. The error from this cause is negligible, since bright interference colours can always be seen as soon as air enters between the faces of the "cup" and "plate" when these are wrung apart after an experiment. The second and third possible sources of error are (a) the volume of the wax used to line the cavity of the "cup", and (b) the volume of grease which will be squeezed into the respiration chamber when it is screwed up tightly in its metal case. No allowance is made for either of these factors, but quantitative investigations showed that from these two causes the results may be anything up to 3% too high.

*Accuracy of the constants.* This was investigated by measuring the volume of  $O_2$  liberated from a known amount of  $H_2O_2$  by means of catalase, control experiments being carried out in the Warburg apparatus. The results are shown in Table I, the calculated values being obtained from titrations with  $KMnO_4$ . The

Table I. *Test experiments with  $H_2O_2$*

In each case approximately 1  $\mu$ l. of gas was evolved in the microrespirometer  
Vol.  $O_2$ /ml.  $H_2O_2$  solution

Exp.	Microrespirometer	Warburg	Calculated
1	204	—	202
	208		
2	198	—	216
	200		
3	78	78 81 81	—
	77		
	77		
	77		
	80		
4	81	77	84.5
	74	77	
	75	78	
	79	79	
	79	80	
5	79	77	82.5
	85	80	
	79	80	
	82	81	
	82	81	

occasional discrepancies are almost certainly due to the fact that the weak  $H_2O_2$  solution decomposes slowly during the equilibration period. Another series of experiments was carried out in which the volume of  $CO_2$ , liberated from bicarbonate solution in an atmosphere containing 5%  $CO_2$  by a known amount of acid, was measured. The results are shown in Table II. The scatter in these experiments is due to the fact that it is difficult to get the "cup", and more particularly the mixing ball, completely free from traces of acid or base. We are convinced that neither of the two sets of figures given is a true indication of the accuracy of the instrument when used for measuring  $O_2$  uptakes.

Table II. *Measurement of volume of CO<sub>2</sub> displaced from bicarbonate by a known amount of acid*

Calculated value, 0.90 $\mu$ l.		
Exp.	Vol. CO <sub>2</sub> evolved $\mu$ l.	% error
1	0.83	-8
2	0.85	-5.5
3	0.90	0
4	0.90	0
5	0.82	-9
6	0.87	-3.5
7	0.84	-6.5
8	0.87	-3.5
9	0.85	-5.5
10	0.90	0
11	0.96	+6.5
12	0.87	-3.5
13	0.89	-1
14	0.88	-2
15	0.89	-1
16	0.92	+2

*The role of diffusion.* In most of the previously mentioned respirometers dealing with uptakes of the order of 1  $\mu$ l. O<sub>2</sub>/hr., shaking is omitted on the assumption that diffusion of the gas in the droplet will be adequate. This point was investigated by comparing the rate of O<sub>2</sub> uptake in the microrespirometer with that in the Warburg apparatus as control. An inorganic system such as a spontaneously oxidizable titanous salt is unsuitable for this purpose, because the rate of O<sub>2</sub> uptake will be proportional to the partial pressure of O<sub>2</sub>; as this will decrease towards the centre of the droplet, the rate of uptake of the inside of the drop will be less than that of the outside layer. What is required is a catalytic system in which the catalyst has a high affinity for oxygen, and experiments showed that kidney amino-acid oxidase with alanine as substrate [Krebs, 1935] was satisfactory in this respect.

A large number of experiments was carried out with this system in an atmosphere of O<sub>2</sub>, with droplets between 4.0 and 16.0  $\mu$ l. in volume, with total uptakes ranging from 0.5 to 2.7  $\mu$ l. O<sub>2</sub>/hr. Of the 59 microrespirometer experiments which were done, the standard deviation of the percentage error was 4.8, a similar scatter being obtained with the Warburg values. The agreement, in any particular series, between the two instruments was in most cases within  $\pm 10\%$ , and though occasionally even worse results were obtained, it is quite clear that in an atmosphere of O<sub>2</sub> there is no effect attributable to diffusion.

Comparative experiments in air, however, showed a definite effect of diffusion, for considerably lower values for the rate of O<sub>2</sub> uptake were obtained with the microrespirometer than with the Warburg apparatus, with drops down to 4.0  $\mu$ l. in volume having an uptake of 0.4  $\mu$ l./hr. Even when the drop was suspended in a platinum loop inside the vessel, so that maximum surface was exposed to the gas mixture, the values were low by as much as 20%.

In view of the results obtained in an atmosphere of air, it was thought desirable to test rates of uptake in O<sub>2</sub> under more stringent conditions. Tyramine oxidase has been shown by Kohn [1937] to be highly dependent on the O<sub>2</sub> pressure, and some comparative experiments with this system were carried out in the microrespirometer, with the Warburg apparatus as control, with the results shown in Table III. The activity of the extract fell off rapidly, so that accurate comparison of the results in the two instruments was not possible. The

Table III. *Experiments with tyramine oxidase*

In all experiments the volume of extract taken in the microrespirometer was 10.3  $\mu$ l., but this quantity was sometimes divided into two or three separate droplets in the respiration chamber.

Exp.	O <sub>2</sub> uptake/hr./ $\mu$ l. extract		Number of drops of fluid in microrespirometer
	Warburg	Microrespirometer	
1	285	306	2
	285	291	1
	303	264	2
		260	1
2	310	291	3
	320	276	3
	280	310	1

table shows clearly, however, that even when oxidative systems are used which are dependent on O<sub>2</sub> pressure, the rates of O<sub>2</sub> uptake of these systems can safely be measured in this instrument. Since correct values are obtained in O<sub>2</sub> with relatively large drops and rapid rates of uptake, it might have been expected that normal values would also be obtained in air, provided that the drop of fluid were sufficiently small. This is not the case and therefore factors other than simple diffusion must be operating to account for this phenomenon. However, a discussion of these other factors is beyond the scope of the present communication.

Comparative experiments, with the Warburg apparatus as control, have also been done on the rate of liberation of CO<sub>2</sub> from urea by the action of urease; entirely satisfactory agreement between the two instruments was obtained.

The apparatus described in this communication is suitable for the measurement of rates of O<sub>2</sub> uptake and other metabolic activities of small pieces of surviving tissues. As, however, the case of small pieces of tissue involves a number of special considerations and the application of special methods, this question is treated in detail in the following paper by Berenblum *et al.* [1939].

Details for the measurement of respiratory quotients and aerobic glycolysis in this instrument will be given in a subsequent paper.

#### SUMMARY

1. A new type of microrespirometer is described which will measure O<sub>2</sub> uptakes of the order of 1  $\mu$ l./hr., with a standard error similar to that normally obtained with the Warburg apparatus.

2. The respiration chamber can be filled with any desired gas mixture, and two or more separate fluids in it may be mixed at any time during the experiment. The apparatus may be charged at one temperature and used at a widely different one, and it can be sterilized.

3. The estimation of substances which can be determined manometrically in the Warburg apparatus can also be carried out in the microrespirometer, the amounts which can be estimated being approximately 200 times smaller.

4. No special skill is required in its manipulation, and the setting up of an experiment takes only slightly longer than in the case of the Barcroft or Warburg apparatus. During the course of an actual experiment six readings can be taken in 5 min., with a little practice.

We wish to thank Dr H. G. Kuhn and Dr P. E. Lindahl for helpful suggestions. Our thanks are due to the Medical Research Council for a personal grant and a grant for apparatus to one of us (N. G. H.), and to the British Empire



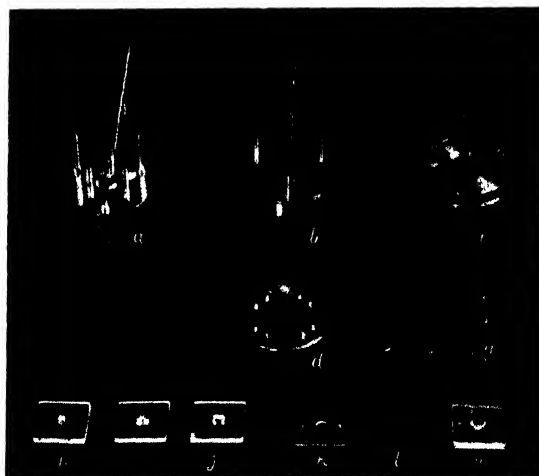


Plate Ia

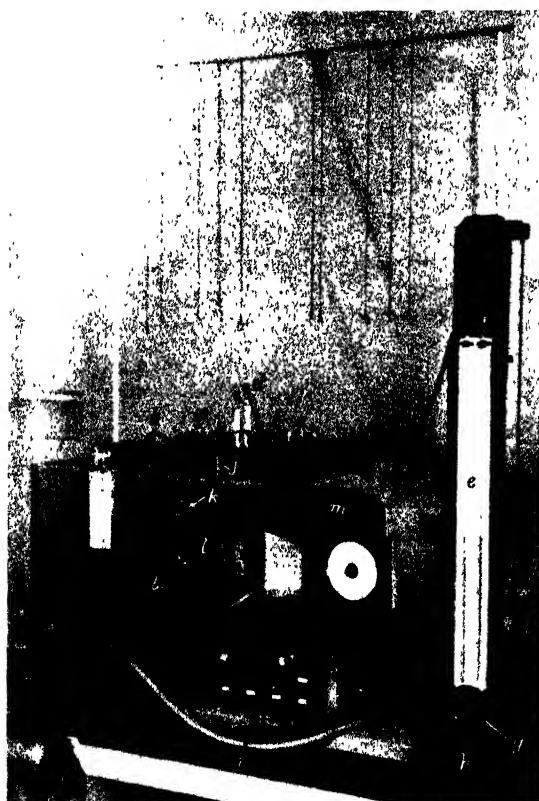


Plate Ib

Cancer Campaign for personal grants to two of us (I. B. and E. C.). Preliminary experiments were carried out by one of us (N. G. H.) at the Biochemical Laboratory, Cambridge, England, during his tenure of the Benn W. Levy studentship.

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## EXPLANATION OF PLATE I

PLATE I<sub>A</sub>

Components of the respiration chamber and brass case. *a*, brass case, face upwards. *b*, brass case, face downwards. *c*, complete assembly ready for immersion in thermostat. *d*, rotating back of case, showing pins. *e*, plain washers. *f*, spring washers. *g*, knurled nuts. *h*, *i* and *j*, "cups" with cavities for two, three and four separate drops of fluid respectively. *k*, "plate". *l*, mica, with mirrors attached. *m*, complete respiration chamber ready for insertion in brass case.

PLATE I<sub>B</sub>

General view of complete apparatus. *a*, thermobarometer. *b*, immersion heaters. *c*, bubblers and manifold leading to manometer, etc. *d*, thermoregulator. *e*, manometer. *f*, one of the reservoirs of the pressure-regulating mechanism. *g*, crank actuating pressure-regulating mechanism. *h*, electromagnet—for mixing drops. *i*, control panel. *j*, top of thermostat stirrer. *k*, window in side of thermostat. *l*, part of optical system (lens, slit and condenser) seen through opened panel of optical box. *m*, hood protecting ground glass screen from stray light.

*Note.* The bar from which the brass cases are suspended is not shown in this photograph.



# IX. THE STUDY OF METABOLIC ACTIVITIES OF SMALL AMOUNTS OF SURVIVING TISSUES

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*(Received 29 October 1938)*

THE microrespirometer described in the previous communication is applicable to the measurement of metabolic activities of small amounts of surviving tissues (e.g. for histochemical studies) and of cell suspensions. In the present communication certain features of technique in micro-manometric studies of the metabolism of minute fragments of surviving tissue are described and consideration is given to special difficulties which arise in such studies.

## *Preparation of tissues for the experiment*

The area of tissue slice which can conveniently be used in the microrespirometer is approximately 0.5–8.0 sq. mm. This size depends upon the metabolic activity of the particular tissue used and must be such as to give a gas exchange of not less than 0.3  $\mu$ l. and not more than 3.0  $\mu$ l. per hr.

The small slices are obtained by cutting a number of ordinary tissue slices from the fresh organ with a sharp razor in the usual manner. One of these, chosen for its thinness and uniformity, is then placed on a microscope slide and cut into small squares with a sharp razor. These tissue pieces are then washed from the slide into a small dish with the required medium and transferred from the dish to the respiration chamber by means of a very fine platinum spatula. Careful handling of these small tissue slices is essential if damage and consequent decrease in the rate of metabolism is to be avoided. The excess medium is removed with a fine capillary and a known amount of fresh medium is added by means of a micro-pipette.

In the case of suspensions of cells such as bacteria, yeast, blood cells etc., a known amount of the suspension is measured directly into the respiration chamber by means of a micro-pipette.

## *Cell damage caused by cutting*

In tissue slices of very small size the ratio of cut surface to total volume is obviously higher than in large slices such as are ordinarily used in the Warburg apparatus. It might therefore be argued that the higher proportion of dead cells (due to the greater amount of cut surface) would result in lower metabolic values being obtained with small than with large slices.

This problem was investigated by comparing in the Warburg apparatus the rates of O<sub>2</sub> uptake of ordinary tissue slices and of similar slices cut up into small squares (as required for the microrespirometer), using liver, kidney and Jensen rat sarcoma for the purpose. The results summarized in Table I show that there is no significant difference between the two series. (The occasional low results with cut up slices of kidney are attributable to a peculiar clumping effect which sometimes occurs when several small pieces of kidney are shaken in the Warburg apparatus).

<sup>1</sup> Beit Memorial Research Fellow.

Table I. *Comparison between the  $Q_{O_2}$  values for whole and cut up slices of different organs (in Warburg apparatus)*

	$Q_{O_2}$ *	
	Whole slices	Cut up slices
Rat liver	8.2	8.0
	9.5	9.3
	8.0	9.0
Rat kidney	17.5	16.0
	16.5	14.0
	18.0	16.3
Jensen rat sarcoma	10.0	9.5

\* The figures represent average values in separate experiments, each figure being the mean of two or more determinations.

The unexpectedly good agreement between the values for whole and cut up slices is due to the fact that a certain proportion of the damaged cells on the surface is removed by the gentle agitation during the preliminary washing and that, though the amount of damaged cells is proportionately greater in the small pieces, the amount shaken off is also proportionately greater. Nitrogen determinations on samples of liquid used for the preliminary washing show that in the case of small slices at least 10% of the initial material is lost in the washings.

### Diffusion

The rate of respiration of most tissue is known to be within wide limits independent of the oxygen tension. It has been shown in the previous communication [1939] that though no provision is made for a shaking arrangement such as that used in the Warburg apparatus, the values for  $O_2$  uptake of homogeneous extracts in an atmosphere of  $O_2$  are the same in the microrespirometer as in the Warburg apparatus, even with oxidative systems which are strongly dependent on  $O_2$  pressure. In an atmosphere of air, however, low values are obtained. Therefore, as far as diffusion of  $O_2$  through the liquid is concerned, it is evident that provided the tests are carried out in  $O_2$  and not in air, the microrespirometer without any stirring can be used safely for the measurement of oxygen uptake of tissue slices. The limiting thickness of the tissue, however, requires separate consideration.

In the respiration chamber, that part of the tissue which touches the bottom does not come into direct contact with the  $O_2$ -containing medium, and under the most unfavourable conditions (i.e. when the whole of one surface is in close contact with the bottom of the cup) almost half of the total surface of the slice will be deprived of direct  $O_2$  supply. It may be assumed, therefore, that the limiting thickness of the tissue which may safely be used in the microrespirometer is, even under the most unfavourable conditions, not less than half of that used in the Warburg apparatus. According to Warburg [1923] in an atmosphere of oxygen the maximal permissible thickness for liver slices is approximately 0.5 mm. and that for kidney slices 0.3 mm. Since it is possible without any special efforts to cut liver slices of 0.25 mm. and kidney slices of 0.15 mm. thickness, no special difficulties need be expected on account of diffusion through the tissue.<sup>1</sup>

<sup>1</sup> The diffusion constant of  $O_2$  through different tissues is not necessarily identical with or even similar to the value for connective tissue of the gut [Krogh, 1919], which has hitherto been generally used in applications of Warburg's formula for the maximal thickness of tissues for metabolic work. For this reason the limiting thickness of any tissue should always be determined by empirical tests.

A comparison between the rates of  $O_2$  uptake of tissue slices measured simultaneously in the Warburg apparatus and in the microrespirometer is given in Table II. The slices were cut without any special precautions, the thinnest and most uniform pieces being chosen for the experiments. (In several cases the thickness of the pieces was estimated from measurement of their areas under the microscope and their weights. No significant differences in the  $Q_{O_2}$  values were observed in the microrespirometer experiments with slices of liver varying in thickness from 0.1 to 0.3 mm.)

Table II. *Comparison between the  $Q_{O_2}$  values for rat liver and kidney obtained in the Warburg apparatus and microrespirometer*

Tissue	$Q_{O_2}$		Weight of tissue used in the microrespirometer $\mu g.$
	Warburg apparatus	Micro-respirometer	
Rat liver	8.3	6.5	173
	8.5	7.1	157
	8.3	5.5	187
	9.2	6.5	208
	8.3	7.4	210
	Av. 8.5	Av. 6.6	
Rat liver	5.2	5.0	187
	5.2	5.0	175
	4.9	4.9	207
	5.3	—	—
	5.4	—	—
	Av. 5.2	Av. 5.0	
Rat liver	13.6	9.5	95
	12.6	9.5	101
	10.3	8.2	93
	10.8	9.4	75
	10.5	—	—
	Av. 11.6	Av. 9.1	
Rat kidney	19.7	18.8	33
	15.5	18.1	45
	—	19.8	24
	—	17.3	37
	Av. 17.6	Av. 18.5	
Rat kidney	17.3	17.7	79
	15.5	20.8	78
	—	15.4	89
	—	18.8	120
	Av. 16.4	Av. 18.2	

Table III. *Effect of oxidizable substrates on  $Q_{O_2}$  values of liver and kidney in the Warburg apparatus and in the microrespirometer*

Tissue	A $Q_{O_2}$ in absence of substrate		B $Q_{O_2}$ in presence of substrate		Ratio: B/A	
	Warburg apparatus	Micro-respirometer	Warburg apparatus	Micro-respirometer	Warburg apparatus	Micro-respirometer
Rat kidney	18.6	16.6	35	27	1.88	1.63
Rat kidney	18.0	18.0	31	29	1.72	1.61
Rat liver	8.0	7.2	13	12.5	1.62	1.73

The substrate used was alanine in the case of kidney and succinate in the case of liver.

Table IV. *Rate of O<sub>2</sub> uptake of yeast suspension expressed as  $\mu$ l. O<sub>2</sub> per ml. suspension per hr.*

Comparison of values obtained in Warburg apparatus and in microrespirometer

Experiment	Warburg apparatus	Microrespirometer
1	170	153
2	200	178
3	213	253
4	136	147

The yeast suspensions were made up as follows: 15 ml. of 1-3% yeast suspension plus 20 ml. of 5% glucose plus 15 ml. of *M*/15 phosphate buffer solution.

Each figure represents the mean of 3-5 determinations carried out simultaneously.

The results show close agreement between the values obtained in the two instruments for the oxygen uptake of kidney slices, but with liver slices in most cases slightly lower values were obtained in the microrespirometer. This is attributable to the fact that liver tissue, being particularly friable, continues to shed dead and damaged cells during the course of the experiment, due to the shaking in the Warburg apparatus, whereas this occurs to a smaller extent with tissues such as kidney. Since these detached cells are not weighed after the end of the experiment, correspondingly high values are obtained with liver slices in the Warburg apparatus as compared with the microrespirometer. The amount of material actually detached was determined by nitrogen estimations of samples from the supernatant fluid in the Warburg vessels taken at different time intervals. It was found to account for approximately 15% of the total weight of tissue. If 15% is added to the  $Q_{O_2}$  values for liver obtained in the microrespirometer (Table II), the values approach those obtained in the Warburg apparatus.

For additional evidence in support of the contention that diffusion through the tissue is adequate, experiments were undertaken on measurements of rates of O<sub>2</sub> uptake of liver and kidney slices with and without oxidizable substrates (succinate for liver and alanine for kidney), the experiments being conducted simultaneously in the Warburg apparatus and in the microrespirometer. The percentage increase in the rates of O<sub>2</sub> uptake of the slices in the presence of substrate showed close agreement in the two instruments (Table III).

Experiments were finally undertaken with yeast suspensions, in order to determine whether, even under these stringent conditions (i.e. a marked tendency for the cells to settle and a very high  $Q_{O_2}$ ), diffusion would still be adequate in the microrespirometer without a shaking system. Once again determinations were made simultaneously in the Warburg apparatus and the microrespirometer, and the results, shown in Table IV, show close agreement in the two instruments.

#### *Determination of amounts of metabolizing material*

(1) *Dry weight.* The metabolic values for tissue slices obtained by means of the Warburg apparatus are usually expressed in terms of unit dry weight. The same standard can be used for the small tissue slices required for the microrespirometer, provided the tissue is sufficiently homogeneous. At the end of the experiment the small tissue slice is carefully removed from the respiration chamber by means of the fine platinum spatula, placed in distilled water for a few seconds (to remove the salts of the medium) and transferred to a tared cover slip. This is dried in an oven at 110° for 1 hr. and reweighed. The ordinary Kuhlmann type microchemical balance can be used for determinations of dry weights of 80  $\mu$ g. or over, but with smaller amounts the weighing error begins

to affect seriously the accuracy of the results. In such cases the Nernst-Donau balance [Donau, 1933] is indicated.

(2) *Nitrogen determination.* As an alternative to dry weight, the N content of the tissue can be used as a standard for expressing metabolic values. Like the dry weight standard, it is only applicable to fairly homogeneous tissues. Since methods of high sensitivity for N determination are now available, the nitrogen standard is applicable to very small amounts of tissue.

(3) *Cell counts.* This has an important theoretical advantage over the previous methods in that it represents a true measure of the respiring elements of the tissue only, so that the variable amounts of non-respiring elements (keratin, collagen, products of secretion etc.) in non-homogeneous tissues will not affect the final results. In practice the cell count standard is applicable to cell suspensions, for which it is probably the ideal method. For tissue slices the method is tedious and troublesome and complicated by the difficulty of making adequate corrections for the cut cells which are counted twice in serial sections. Such a method has, however, been successfully worked out for certain cases by Linderstrom-Lang *et al.* [1935].

(4) *Nucleic acid standard.* When using tissues such as skin, thyroid, brain, necrotic tumour, tissue culture etc., in which large and variable amounts of metabolically inactive material are present, the dry weight and nitrogen standards are unsuitable, since they do not differentiate between the cellular respiring elements and the inactive non-respiring material. The metabolic values, based on such standards, are not only abnormally low but also grossly irregular, since the proportion of living to non-living material in such tissues is liable to vary under different conditions of health and disease and from one animal to another.

The standard required for expressing metabolic activities of such tissues is one which differentiates cellular from non-cellular elements. The nucleic acid content of the tissues appears to answer this purpose.

The method which has been used by the authors consists of the estimation of nucleic acid phosphorus.

*Method.* After removal from the respiration chamber, the piece of tissue is placed in a small tube of about 5 ml. capacity, containing about 2 ml. of an alcohol-chloroform mixture (3:1), and heated for 2 hr. under a small reflex condenser similar to that described by Wasitzky [1932]; this extraction is repeated with fresh alcohol-chloroform mixture for a further 2 hr. The purpose of this is to remove lipid phosphorus. The tissue is then extracted with *N*/10 HCl in the cold for 3 hr., to remove the inorganic and organic acid-soluble phosphorus. For efficient extraction, continuous agitation is essential, and this is conveniently carried out by mounting the stoppered tubes radially on a vertical disk which is slowly rotated by a motor. The tissue is finally ashed with perchloric acid and the phosphorus estimated by the micro-method of Berenblum & Chain [1938].

The method gives reasonably accurate results with amounts of nucleic acid phosphorus down to 0.2  $\mu\text{g.}$ , representing approximately 1.8  $\mu\text{g.}$  of nucleic acid. Table V shows some values for the nucleic acid phosphorus contents of liver, kidney and brain obtained by this method. Fujiwara *et al.* [1937] found the nucleic acid phosphorus content of rat's liver to be 0.434 %, while Javillier & Allaire [1926] give the following figures for horse's tissue: liver=0.204 %, kidney=0.169 % and brain=0.075 %.

Estimations of nucleic acid phosphorus were also carried out on special tissues with the object of examining the suitability of nucleic acid as a measure of cellular material.

Table V. *Nucleic acid phosphorus contents of liver, kidney and brain of the rat*

Tissue	Nucleic acid phosphorus content, expressed as % of dry wt.
Liver	0.27, 0.37, 0.38, 0.36, 0.32 Av. 0.34
Kidney	0.30, 0.29, 0.27, 0.31, 0.29 Av. 0.29
Brain	0.09, 0.14, 0.10, 0.12, 0.11, 0.11 Av. 0.11

The estimations were carried out on small pieces of tissue varying from 216 to 885  $\mu$ g. in dry wt.

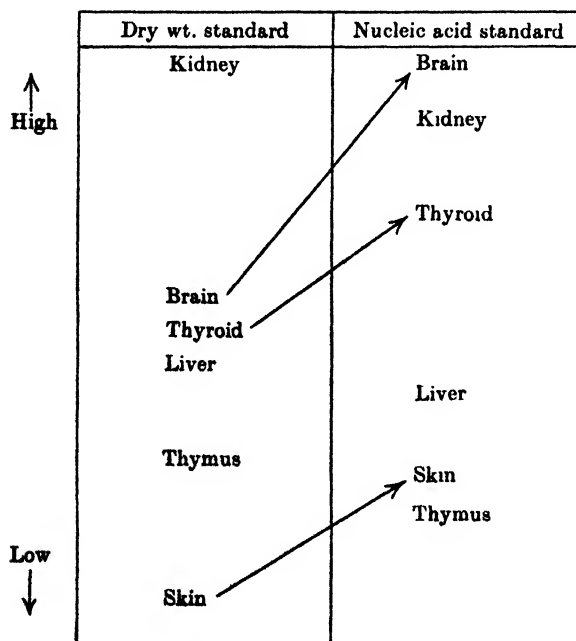
 Table VI. *Nucleic acid phosphorus contents of tissues undergoing autolysis*

Tissue	Nucleic acid phosphorus content (as % of dry wt.)
Jensen rat sarcoma (non-necrotic portions)	0.70, 0.63, 0.84, 0.52, 0.70, 0.68 Av. 0.68
Jensen rat sarcoma (necrotic portions)	0.15, 0.18, 0.19, 0.24, 0.20 Av. 0.19
Liver (normal)	0.31, 0.35, 0.32 Av. 0.33
Autolysing liver* after 24 hr.	0.19, 0.15, 0.15 Av. 0.16
Autolysing liver* after 2 days	0.19, 0.16, 0.19 Av. 0.18
Autolysing liver* after 5 days	0.20, 0.17, 0.21 Av. 0.19
Tuberculous caseation from spleen of monkey	0.34, 0.37, 0.33, 0.34, 0.37, 0.37 Av. 0.35

\* Thin slices of rat's liver floating in saline (with addition of a little toluene) kept at 37°.

Pure connective tissue, as exemplified by tendon of the rat's tail, was found to contain no significant amount of nucleic acid phosphorus. In order to investigate the rate of disappearance of nucleic acid following the death of a cell, three different examples of necrotic tissue were chosen for investigation, namely autolysing liver, the necrotic part of a tumour and tuberculous caseation. The results of these tests (Table VI) can be briefly summarized as follows.

The necrotic part of the tumour contained about one quarter of the nucleic acid of non-necrotic tumour; in autolysing liver the nucleic acid content fell off rapidly to about half of the original content and then remained fairly steady; in caseating material the nucleic acid content was found to be high.

 Table VII. *Diagram showing order of the relative metabolic values ( $Q_{O_2}$ ) of different tissues based on dry wt. and nucleic acid standards*


The results may be explained by the fact that the enzymes responsible for the disappearance of nucleic acid are themselves ultimately destroyed during the process of autolysis. In caseation, where coagulation of the whole cell content sets in quickly, the nucleic acid splitting enzymes are inactivated very early, so that the bulk of the nucleic acid remains intact.

The metabolic values, quoted in the literature [cf. Krebs, 1933], for slices of tissue, have hitherto been calculated in terms of dry weight. Since on this standard no allowance is made for inactive elements in the tissue, the values obtained cannot be expected to bear any relation to the true metabolic activity of the cells contained therein. This is borne out by the comparison between the relative metabolic values ( $Q_{O_2}$ ) for different tissues based on dry weight and on nucleic acid standards (see Table VII).

From general considerations of the physiological functions of these organs and from their respective blood supplies in the intact body, the order of their metabolic activities would seem to correspond much more closely to that given by the nucleic acid standard than that based on dry weight.

#### SUMMARY

1. Details of the cutting and preparation of small tissue slices, suitable for use in the microrespirometer, are described.

2. Diffusion of  $O_2$  through the tissue was found to be adequate, provided the slices were cut reasonably thin.

3. In the Warburg apparatus tissue slices which had been cut into small pieces gave identical  $Q_{O_2}$  values with whole slices.

4. Comparative experiments on the  $O_2$  uptake of liver and kidney slices, carried out simultaneously in the Warburg apparatus and microrespirometer, showed reasonably good agreement between the two, both in the presence and absence of oxidizable substrates. Thus, under suitable conditions, the effects of diffusion and of damage of the tissue due to cutting, are negligible.

5. Various methods for determining the amounts of metabolizing material are discussed. For tissues containing large amounts of inactive material (e.g. skin, thyroid, necrotic tumour, tissue culture etc.), it is suggested that the metabolic values be expressed in terms of nucleic acid phosphorus content. A convenient method for the estimation of nucleic acid phosphorus in small amounts of tissue is described.

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# X. THE IMMUNOLOGICAL PROPERTIES OF PROTEINS TREATED WITH $\beta\beta'$ -DICHLORO-DIETHYLSULPHIDE (MUSTARD GAS) AND $\beta\beta'$ -DICHLORODIETHYLSULPHONE<sup>1</sup>

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SEVERAL explanations have been put forward to account for the vesicant action of mustard gas and related compounds. Cashmore & McCombie [1923] refer to the suggestion that the effect might be due to a reaction between the mustard gas and "amino-acids present in the skin". In support of this theory might be mentioned the experiments of Clarke [1912], who showed that mustard gas combines with primary amines, those of Cashmore & McCombie [1923], who found that mustard gas and  $\beta\beta'$ -dichlorodiethylsulphone give condensation products with glycine ester, and the observation that  $\beta\beta'$ -dichlorodiethyl-sulphide, -sulphoxide and -sulphone react with various primary, secondary and tertiary amines [Lawson & Reid, 1925]. The last-named authors conclude that their results are consistent with the theory that the vesicant action of mustard gas is due to its reaction with constituents of the living cell.

The introduction of new groups into the protein molecule, by attachment to the free amino-groups, leads usually to a very marked change in the immunological properties of the protein. Landsteiner & Jablons [1914] and Landsteiner & Lampl [1917] found that treatment with formaldehyde renders rabbit serum proteins fully antigenic for rabbits. Although the original species specificity is not completely destroyed, the action of formaldehyde is sufficient to produce a protein derivative which is "foreign" to the body. Horsfall [1934] obtained essentially similar results with formaldehyde-treated proteins.

Amongst other reagents which react with the free amino-groups of proteins is phenyl isocyanate, and this substance destroys, almost completely, the original species specificity of serum proteins; the phenylcarbamido-proteins produced have an immunological specificity which is characteristic for the phenylcarbamido-grouping [Hopkins & Wormald, 1933, 1, 2: 1934]. Similar results follow the action of benzyl chloroformate on antigenic proteins [Gaunt *et al.* 1935; Gaunt & Wormald, 1938], for in this case also the acquirement of a new immunological specificity is related to the introduction of a new group attached to the free amino-groups of the original protein.

<sup>1</sup> The investigations described here were mainly carried out when the authors were members of the Departments of Experimental Pathology and of Physiology in the University of Leeds. A brief account of the earlier results was communicated in 1936 to the 2nd Internat. Congress for Microbiology (cf. *Report*, 1937, p. 427).

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In view of these immunological investigations and the above-mentioned work on the action of mustard gas and related compounds on amino-acids, it was considered desirable to study the immunological properties of mustard gas-treated proteins. The investigations described were undertaken (a) to determine whether serological methods would enable us to detect a reaction between mustard gas and proteins, and (b) to study the general immunological properties of these mustard gas- (and sulphone)-treated proteins.

#### EXPERIMENTAL

For the preparation of mustard gas- (or sulphone)-treated proteins, the reagent was allowed to act on the protein solution in the presence of phosphate buffer at pH 8.0. Since mustard gas is gradually hydrolysed by water with liberation of free HCl, occasional addition of 2 N NaOH was sometimes necessary to maintain the pH at about 8. After some time the mixture was extracted with ether to remove unchanged mustard gas and the resulting solution used for immunization or for the serological tests. No attempt was made to separate the altered proteins from the mixture, since the sole object of early studies was to determine whether any change had occurred which could be detected immunologically.

*Mustard gas-treated proteins.* A mixture of 100 ml. of serum, 100 ml. of a phosphate buffer of pH 8.0 (100 ml. of 0.2 M  $\text{KH}_2\text{PO}_4$  plus 93.6 ml. of 0.2 N NaOH) and 1 g. of NaCl was stirred at room temperature for 8 hr. on each of three successive days, after the addition of 2 ml. of mustard gas on the first and second days. During each night the mixture was kept in the ice-chest. It was finally extracted five times with 200 ml. of ether each time, the ether removed by evacuation and the pH adjusted to 7.5. The preparations used for immunization were treated with 0.05 vol. 5 % phenol.

*"Sulphone"-treated proteins.* These were prepared in the same way as were the mustard gas-treated proteins but the solid  $\beta\beta'$ -dichlorodiethylsulphone was added in ethereal solution (two additions of 2 g. of the sulphone in about 45 ml. of ether to 100 ml. of serum). In other experiments the reaction was carried out at 30° instead of at room temperature, and the two preparations are referred to in this paper as sulphone-treated proteins (17°) and sulphone-treated proteins (30°).

*Immunization.* The antigens injected were all prepared from normal horse serum. The method of immunization was that recorded previously [Hopkins & Wormald, 1933, 1], all the injections being made intraperitoneally. The injected rabbits were bled after 3-7 injections. The serological reactions mentioned below were often observed after the third injection but a better response was obtained after six or seven injections.

*Precipitin tests.* These tests were carried out as described by Hopkins & Wormald [1933, 1].

*Complement-fixation tests.* Mixtures of 0.5 ml. of diluted antigen, 0.10 ml. of the antiserum (previously heated at 56° for 20 min.) and 0.10 ml. of 1:5 guinea-pig serum (in 0.9 % NaCl) were kept at room temperature for 1 hr. To each tube was then added 0.5 ml. of a 4 % suspension of sensitized ox red cells (in 0.9 % NaCl) and the tubes were immersed in a water bath at 37°. Readings were taken at various intervals. The results given in Table II are typical results obtained after 1 hr.

*Results of the serological tests*

The injection of mustard gas-treated horse serum into rabbits results in the production of antibodies which give precipitin reactions with the antigen injected and to a lesser extent with mustard gas-treated proteins of rabbit serum (or chicken serum). The reactions observed (Table I) were sufficiently pronounced to establish definitely that a new specificity had been acquired. The retention of the original species specificity of the serum proteins, shown by the reactions between horse serum and antisera to mustard gas-treated horse serum and between mustard gas-treated horse serum and antisera to horse serum, is not unexpected since the mustard gas-treated protein preparations undoubtedly contained unchanged serum proteins. In view of the possibility that the precipitin reactions with the mustard gas-treated proteins might be related to groups substituted in the free amino-groups of the protein, cross-tests were made with phenylcarbamido-proteins (proteins in which the free amino-groups have been replaced by  $\text{NH}\cdot\text{CO}\cdot\text{NH}\cdot\text{C}_6\text{H}_5$ ). No cross-reactions were observed, and this is perhaps not surprising in view of the sharp specificity observed when new groups are introduced into protein molecules (for a review of the literature cf. Landsteiner [1936]; Marrack [1938]). Confirmation of the reactions between antisera to mustard gas-treated horse serum and mustard gas-treated rabbit serum was obtained by complement-fixation tests, representative results of which are given in Table II.

Table I. *Precipitin reactions with antisera to mustard gas-treated proteins*

Antigen	Dilution of antigen	Immune serum against				Phenyl-carbamido-horse serum proteins No. 100
		Mustard gas-treated horse serum			Horse serum No. 82	
		No. 498	No. 198	No. 199		
Horse serum	1:20	+	±	tr.	++	-
	1:100	++	+	±	++	-
	1:500	+ ±	+	±	++	-
	1:2500	+ ±	±	±	±	-
Mustard gas-treated horse serum	1:20	++	++ ±	+ ±	++	-
	1:100	+++	++ ±	+ ±	++	-
	1:500	++	+ ±	+	++	-
	1:2500	±	±	±	±	-
Mustard gas-treated rabbit serum	1:20	-	-	-	-	-
	1:100	±	±	±	-	-
	1:500	±	+	+	-	-
	1:2500	tr.	±	tr.	-	-
Mustard gas-treated chicken serum	1:20	-	-	-	-	-
	1:100	-	f. tr.	f. tr.	-	-
	1:500	±	±	±	-	-
	1:2500	f. tr.	f. tr.	f. tr.	-	-

The dilutions of antigen in Tables I, II and III refer to dilution of a solution which contains 5% of total protein. Precipitin test readings (taken after 1 hr. at 37°): - (no reaction), f. tr. (faint trace), tr. (trace), ±, +, + ±, ++ etc., in increasing degrees of precipitation.

Similar results were obtained with the proteins treated with  $\beta\beta'$ -dichlorodiethylsulphone. Precipitin tests (Table III) and complement-fixation tests showed that antisera to the sulphone-treated proteins are capable of reacting with sulphone-treated rabbit serum; they will not react, however, with mustard gas-treated rabbit serum. In these experiments the best reactions were given with the preparations obtained by allowing the sulphone to act on the proteins at 30°,

Table II. *Complement-fixation tests*

Antigen dilution	Antigen	Immune serum	Mustard gas-treated horse serum		Mustard gas-treated rabbit serum	
			No. 498	No. 198	No. 498	No. 198
1:20			3	2	4	4
1:60			1	1	3	4
1:180			—	—	3	2
1:540			—	—	2	—
1:1,620			—	—	1	—
1:4,860			—	—	—	—
1:14,580			—	—	—	—
1:43,740			—	—	—	—
1:131,220			—	1	1	—
1:393,660			1	3	2	1
1:1,180,980			2	4	4	2
1:3,542,940			4	4	4	4
Control (NaCl)			4	4	4	4

Readings: 4 (complete haemolysis); — (no haemolysis); 3, 2 and 1 intermediate degrees of haemolysis.

Antisera: Nos. 498 and 198 (antisera to mustard gas-treated horse serum).

and it seems probable that at body temperature the chemical reaction between proteins and mustard gas (or the corresponding sulphone) may be appreciably quicker than that observed in the experiments recorded here. Further experiments are now being carried out to determine the minimum time required for mustard gas and the sulphone to produce at 37° a change in the protein molecule which can be detected by these serological methods.

Table III. *Precipitin reactions of antisera to "sulphone"-treated proteins*

Antigen	Dilution of antigen	Immune serum against				Mustard gas-treated horse serum No. 493
		Sulphone-treated horse serum				
		No. 403	No. 404	No. 406	No. 408	
Horse serum	1:20	tr.	tr.	—	—	tr.
	1:100	±	±	±	tr.	+
	1:500	tr.	tr.	tr.	tr.	±
	1:2500	—	tr.	f. tr.	—	tr.
Sulphone-treated horse serum (30°)	1:20	++	++ ±	±	+	f. tr.
	1:100	++	++ ±	+	+	±
	1:500	+	+ ±	+	+	+
	1:2500	tr.	±	tr.	tr.	tr.
Sulphone-treated rabbit serum (30°)	1:20	—	tr.	—	—	—
	1:100	tr.	+	f. tr.	f. tr.	—
	1:500	+	+	f. tr.	tr.	—
	1:2500	+	+	tr.	+	—

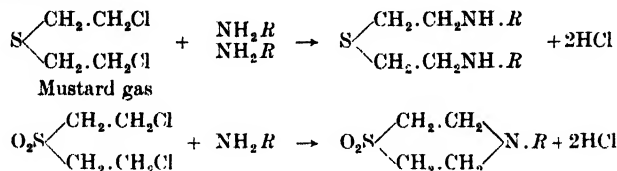
Immune sera nos. 403 and 404 were obtained with the sulphone-treated horse serum (30°) and nos. 406 and 408 with sulphone-treated horse serum (17°).

## DISCUSSION

The results of the experiments described here show that mustard gas and  $\beta\beta'$ -dichlorodiethylsulphone effect a change in proteins which can be detected by immunological methods. The change is sufficient to convert the protein into derivatives which may be considered "foreign" to the homologous animal. Some of the more characteristic features of the vesicant action of mustard gas are the smallness of the effective dose, the delayed action and the fact that people who have been badly "gassed" are liable to become hypersusceptible to mustard gas.

These facts are compatible with the view that the biological mechanism of the reaction may be related to protein sensitivity, but whether the present results have any bearing on this problem cannot be decided without further investigation.

The identity of the chemical changes effected in the protein molecule by mustard gas and the sulphone has not yet been established, but it seems probable that they are concerned with the free amino-groups of the protein. Cashmore & McCombie [1923], using glycine ester, obtained products which suggest reactions of the following types:



Lawson & Reid [1925] obtained similar results but found that although amino-acids condense readily with the sulphone they do not react with the sulphide (mustard gas). In these experiments of Cashmore & McCombie and those of Lawson & Reid, the conditions of the reaction were much more drastic than those employed by us. In the investigations described here, however, it has been necessary to avoid high temperatures and the use of strong alkalis or acids, in order that the antigenic power of the proteins might not be destroyed. Furthermore, it was the intention to reproduce as far as possible reactions which might take place when mustard gas is allowed to come into contact with the animal body. In spite of these differences it seems probable that the protein changes detected by the serological tests are similar to those occurring in amino-acids and their esters. The differences between the actions of mustard gas and of  $\beta\beta'$ -dichlorodiethylsulphone [Cashmore & McCombie, 1923] would readily explain the failure, in our experiments, of proteins treated with mustard gas and those with the sulphone to give serological cross-reactions (cf. Table III).

In connexion with these studies of the action of mustard gas on proteins there are several other observations which appear to be pertinent. Arising out of the work on the anticarcinogenic action of mustard gas [Berenblum, 1935], Berenblum *et al.* [1936] studied the effects of mustard gas and  $\beta\beta'$ -dichlorodiethylsulphone on the respiration and glycolysis of minced Jensen sarcoma tissue *in vitro*. They found that these compounds reduce the glycolysis of the tumour material more than its respiration. Jány & Sellei [1935] also noted that mustard gas inhibits glycolysis by *B. coli* and by Ehrlich rat sarcoma. Peters [1936] has shown that  $\beta\beta'$ -dichlorodiethylsulphone has an inhibitory effect, similar to that of iodoacetic acid, on the respiration of brain tissues: this inhibition is exercised on the change pyruvate  $\rightarrow$  oxidation products, and the sulphone has no appreciable effect on the conversion, in this tissue, of lactate into pyruvate. The inactivation of certain enzymes in the tissues studied by these various authors may possibly be due to the same change which produces an alteration in the immunological properties of the protein. Investigation from both aspects may, therefore, throw some light on the nature of the action of mustard gas and related compounds on the animal body.

#### SUMMARY

1.  $\beta\beta'$ -Dichlorodiethylsulphide (mustard gas), when allowed to act on horse-serum proteins at room temperature, produces a change in the immunological properties of these proteins. These derivatives produce in the rabbit antibodies which are capable of reacting with mustard gas-treated rabbit serum.

2.  $\beta\beta'$ -Dichlorodiethylsulphone-treated horse-serum proteins produce antibodies which will react with sulphone-treated serum proteins, but not with the mustard gas-treated proteins.

3. The possible chemical basis of this change in immunological properties may be an interaction between the mustard gas (or sulphone) and the free amino-groups of the protein molecule.

The authors would like to take this opportunity of thanking the Medical Research Council for a grant to one of them (A. W.) to cover part of the expenses incurred in this work.

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# XI. STUDIES ON BAYER 205 (GERMANIN) AND ANTRYPOL<sup>1</sup>

## III. FURTHER OBSERVATIONS ON THE METHOD OF DETERMINATION AND ON THE RETENTION OF THIS DRUG IN THE ANIMAL BODY

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IN a previous communication [Dangerfield *et al.* 1938] a method for the determination of Bayer 205 and Antrypol in the blood plasma was described, and experimental evidence was produced to show that after the injection of a "normal" dose of Bayer 205 into a rabbit or dog there is a significant amount of the drug in the plasma 3 months or even longer after the injection. Further investigations have been concerned (a) with the simplification of the method (by the use of a Lovibond comparator) so that Bayer 205 determinations on blood plasma, C.S.F. and possibly other body fluids might be possible in tropical countries when a colorimeter is not available, and (b) with a modification of the method for determinations on urine. The observations on the retention of the drug in the body have also been extended in order to provide a continuous curve to show the amount of Bayer 205 in the plasma of rabbits during and after a series of injections of the drug. During these investigations no appreciable difference has been observed between Bayer 205 and Antrypol, and in most of the later experiments the latter preparation has been used.

### *Method of determination*

It has not been found necessary to make any significant alteration in the method described in the earlier paper, but one or two slight improvements have been introduced. The hydrolysis by conc. HCl is now effected in glass-stoppered pyrex tubes graduated at the 10 ml. level. At one period in these investigations difficulty was experienced in matching the final colours, owing to occasional development of turbidity following the addition of the methyl- $\alpha$ -naphthylamine solution. It was found that this turbidity was due to impurities (possibly Cu) in the distilled water, and this trouble was overcome by passing the distilled water twice through a permutit water-softener.

Subsequent work has fully confirmed the suggestion that methyl- $\alpha$ -naphthylamine hydrochloride has many advantages over the free base when used as the coupling agent. The free base (an oil) develops a red colour fairly rapidly whereas the solid hydrochloride shows very little change in colour over a long period, particularly if exposure to sunlight is reduced to a minimum. The solution used in the actual determination (0.2 g. in 100 ml. in 50% v./v. acetic acid) shows very little reddening in 1 or 2 weeks if kept in a dark bottle.

<sup>1</sup> This communication represents a second report to the Medical Research Council at whose request this work was undertaken.

*Preparation of methyl- $\alpha$ -naphthylamine hydrochloride.* The preparation of the free base was essentially that described by Fischer [1895]. The following additional details of the method might prove useful.  $\alpha$ -Naphthylamine was formylated [Tobias, 1882], and the product recrystallized and then methylated. For the methylation the xylene should be perfectly dry and granulated sodium should be used. After the addition of the sodium, a vigorous reaction occurred and a white precipitate formed; the mixture was cooled to  $80^{\circ}$ , methyl iodide added and the temperature raised to  $100$ – $110^{\circ}$ , with subsequent completion of the reaction by boiling the mixture under reflux for 1 hr. The cooled product was filtered and the filtrate was treated with charcoal and freed from xylene by steam distillation. The formylmethyl- $\alpha$ -naphthylamine was hydrolysed by boiling for 1 hr. under reflux with 5 vol. dil.  $\text{H}_2\text{SO}_4$  (10 % v./v.), the solution filtered and treated with charcoal, and the final filtrate kept overnight, when a little  $\alpha$ -naphthylamine separated out. The filtered solution was basified with NaOH, and the base extracted with ether. The ethereal solution was treated with charcoal and dried with  $\text{CaCl}_2$ . Dry HCl was passed into this solution to precipitate methyl- $\alpha$ -naphthylamine hydrochloride. The precipitate obtained in the early stages contained some violet impurity and was discarded. The main bulk precipitated by further passage of HCl was dried *in vacuo* in the presence of solid KOH. No further purification by recrystallization was possible but this preparation, which had only a very faint pink tinge, was pure enough for the purposes of this investigation. The yield was 12.8 g. of the hydrochloride from 14 g. of crude base, and a similar yield was obtained from a sample of the base supplied by Messrs May and Baker, Ltd., Dagenham.

*The use of a Lovibond comparator.* At the suggestion of Dr F. Hawking, who wished to carry out determinations of Bayer 205 in East Africa, coloured discs have been made for this purpose by "The Tintometer Ltd." A set of two discs covers the ranges equivalent to 0.18 to 1.20 and 1.20 to 3.60 mg. of hydrolysed Bayer 205 per 100 ml. of solution to be diazotized. The matching of the colours is quite easy and can be done with accuracy up to the reading of 2.70. With larger amounts of Bayer 205 it is advisable to use a smaller volume (i.e. less than 2 ml.) of the hydrolysed solution and to add 3 N HCl to give 2 ml. for the diazotization process. When these discs are used it is possible to determine with satisfactory accuracy amounts of Bayer 205 greater than 0.5 mg./100 ml. of plasma or other fluid. On the rare occasions when smaller amounts have to be determined, the solution should be compared with a set of standards.

The comparator method has several advantages over the colorimeter method. Normal plasma and sera contain a small amount of diazotizable substances which can easily be allowed for by the use of the control tube, and this compensation for "blank values" has proved of considerable value for the determination of Bayer 205 in urine and certain animal tissues. When a "blank" of this type is not available, compensation for any yellow or brownish colour in the filtered hydrolysates can be made by using, as a control, a mixture of the filtrate and all the other reagents except the sodium nitrite.

#### *Products of acid hydrolysis of Bayer 205*

Lang [1931] states that the hydrolysis of Bayer 205 by hot HCl yields *m*-aminobenzoic acid, *m*-amino-*p*-toluic acid and  $\alpha$ -naphthylamine-4:6:8-trisulphonic acid, but there appears to be no evidence that the disruption of the complex molecule of the drug is as complete as this. For another aspect of our investigations these three amines are required, and the opportunity has been taken to determine whether a mixture of the three would give the same colour

when diazotized and coupled with methylnaphthylamine as does an equivalent amount of hydrolysed Bayer 205. The colour obtained with an equimolecular mixture of these amines was approximately as intense as, but definitely more purple than, the colour with hydrolysed Bayer 205. The results suggest that a large part of the colour with the last-named is due to *m*-aminobenzoic acid. Each of the other two amines gives a lilac colour and these observations tend to show that hydrolysis with HCl is not as complete or simple as was suggested by Lang. This point is perhaps of theoretical interest only at present, and does not affect in any way the method of determination of Bayer 205, but it may be of significance when the possible decomposition of this drug in the body is studied.

#### *Determination on serum*

For some purposes it may be necessary to use serum for these determinations, and a comparison has been made of the values obtained with the plasma and sera of rabbits which had previously received injections of Antrypol. Blood was collected from the ear of each rabbit in amounts of 2.5, 5.0 and 2.5 ml. The first and third samples were collected in oxalated tubes and were then mixed and centrifuged for the separation of the plasma. The second blood sample was allowed to clot and the serum obtained. Bayer 205 determinations were made, and the results recorded in Table I show that the plasma and serum from blood containing Bayer 205 contain the same amount of this drug.

Table I. *Bayer 205 (or Antrypol) determinations on plasma and serum*

	mg. Antrypol per 100 ml.	
	Plasma	Serum
Rabbit 493 (Antrypol)	3.50	3.60
" 494 (Antrypol)	3.40	3.40
" 495 (Control)	0.95	1.00
" 496 (Control)	0.95	0.95

The results in this table have not been corrected for the blank value of approximately 1.0 mg./100 ml. for normal rabbit plasma or serum.

#### *Determinations on urine*

In an earlier paper [Dangerfield *et al.* 1938] it was mentioned that the method devised for the determination of Bayer 205 in plasma cannot be used for determinations of the drug in urine because of the presence in normal urine of a very variable amount of diazotizable substances. The necessary blank values may be as low as 0.25 or as high as 5.0 mg./100 ml. (calculated as hydrolysed Bayer 205), and it is not possible by any simple method to compensate for this blank value. There is no satisfactory evidence as yet that unchanged Bayer 205 is excreted in the urine, but the excretion in the urine of a trypanocidal agent after the injection of the drug [Mayer & Menk, 1921; 1922; Thomson & Robertson, 1922] and some preliminary observations recorded earlier in the present investigations [Dangerfield *et al.* 1938] suggest that this excretion does take place. Further investigations which will be reported later leave no doubt that Bayer 205 either in the original or slightly modified form, is excreted in the urine in fairly appreciable amounts for a few days after the injection.

Since the use of a blank value for normal urine is precluded by the wide variation in this value, efforts have been made to destroy the "normal" amines without loss of Bayer 205, but without success. Ultimately it was found possible, however, to separate these normal amines (or "amine-precursors") from Bayer



205 by ultrafiltration through a standard collodion membrane, and this is the basis of the method now adopted. Collodion membranes are prepared by the method described by Folley [1933] and are dried for 15 min. in a current of air passing through the apparatus at the rate of 2 l. per hr. at room temperature (19–21°). The membranes are then immersed in distilled water for 24 hr., and filtration is effected with a positive pressure of 120 mm. Hg in the apparatus described by Folley. Normal urine (of man or dog) yields an ultrafiltrate which, on hydrolysis with HCl, gives the same amount of diazotizable amine as does the untreated urine, but no Bayer 205 passes through the membranes. If determinations are made, therefore, on a urine containing Bayer 205 and on an ultrafiltrate from this urine, the difference between the two values is a measure of the Bayer 205 present.

The "recovery" of Bayer 205 when added to urine is, however, not quantitative, but usually varies between 70 and 95 % with an average of about 85 %. (With one sample of urine, however, the recovery was a little below 50 %.) This loss is fairly constant for any given sample of urine, e.g. with the same urine the loss may be 15 % with amounts of Bayer 205 varying from 1 to 10 mg./100 ml. (cf. Table II). A fairly satisfactory estimate of the true Bayer 205 content of the urine can thus be obtained by the use of a Bayer 205 "recovery factor" for the urine concerned, this factor usually being between 1.10 and 1.25. For a true estimate of the amount of Bayer 205 in any sample of urine it is necessary therefore, to carry out determinations on (a) the urine, (b) an ultrafiltrate from the urine, prepared as described above and (c) the urine treated with a known amount of Bayer 205 (e.g. 2 or 3 mg./100 ml. of urine). If (c) is prepared by the addition of a small amount of strong Bayer 205 solution to the urine, the urine for (a) and (b) should be diluted with a corresponding amount of water. The difference between (a) and (b) will give the minimum Bayer 205 content of the urine (or diluted urine), and a more correct value is obtained by multiplying this value by a factor if the difference between (a) and (c) shows that the recovery is not complete. With the Lovibond comparator method these differences can be measured very readily since the less-coloured sample can be placed in the control tube. (A control determination is, of course, made on the Bayer 205 solution used for the preparation of (c), and in all the experiments quoted here duplicate determinations were made.)

Several possible explanations for this incomplete recovery of Bayer 205 added to urine have been examined, in the hope that some slight modification might render the use of a recovery factor unnecessary. A longer period of hydrolysis (8 hr. instead of 6 hr.) or an increase in the amount of HCl, does not increase the yield. One of the factors concerned is possibly the presence in hydrolysed urine of substances which prevent the maximum colour development, e.g. the liberation of phenolic substances from ethereal sulphates and the combination of these phenols with the diazotized amines. The addition of hydrolysed urine to a separately hydrolysed Bayer 205 solution causes only a slight loss of amine (about 5 %), and this explanation can account for only part of the total loss. It was ultimately decided that it is wiser to adopt a correction factor in these investigations rather than attempt to devise a complicated technique which may lead to greater inaccuracies.

Some of the results of determinations on normal urines after the addition of Bayer 205 are recorded in Tables II and III, and from these results it will be seen that the method can be used for the detection and determination of small amounts of Bayer 205 or Antrypol. Amounts greater than 0.6 mg./100 ml. of urine can be determined with satisfactory accuracy. A chemical method of this type cannot,

Table II. *Recovery of Bayer 205 added to normal urine*

Subject	Amount of Bayer 205 added	"Amine" plus Bayer 205 mg. Bayer 205/100 ml.	Bayer 205	Recovery %
1	0	0.66	—	—
	1.0	1.48	0.82	82
	2.0	2.33	1.67	83
	3.0	3.07	2.41	80
	5.0	4.67	4.01	80
2	0	1.93	—	—
	0.97	2.66	0.73	75
	3.10	4.33	2.40	77
3	0	0.70	—	—
	0.95	1.60	0.90	95
	3.0	3.27	2.57	86
	11.0	10.0	9.30	85
4	0	0.77	—	—
	1.0	1.59	0.82	82
	3.2	3.33	2.56	80
	10.7	10.20	9.43	88
5	0	0.67	—	—
	1.00	1.60	0.93	93
	3.0	3.43	2.76	92
	10.3	10.40	9.73	94
6	0	0.73	—	—
	1.00	1.20	0.47	47
	2.20	1.62	0.89	40
	4.50	2.68	1.95	43
	7.85	4.13	3.40	43

The above results are average values of two or three determinations (i.e. separate hydrolyses). The urines for these determinations and for those given in Table III were diluted with one-ninth their volume of water or Bayer 205 solution (of ten times the strength recorded in column 2).

Table III. *Determination of Bayer 205 (or Antrypol) in urine*

Amount of Bayer 205 added to urine	Bayer 205 plus "amine"		Bayer 205 value	Corrected* Bayer 205 value
	Untreated urine	Urine ultrafiltrate		
2.0	2.59	0.98	1.61	2.07
0	1.61	1.60	—	—
1.0	2.47	1.55	0.92	1.13
3.0	4.00	1.57	2.43	2.99
0	1.80	1.78	—	—
1.0	2.50	1.72	0.78	1.08
3.0	4.00	1.80	2.20	3.04
0	0.25	0.25	—	—
1.86	1.80	0.25	1.55	1.89
3.95	3.33	0.25	3.08	3.76
5.87	5.08	0.25	4.83	5.89
0	0.72	0.70	—	—
2.17	2.59	0.72	1.87	2.19
4.43	4.60	0.70	3.90	4.56
6.67	6.40	0.72	5.68	6.65

\* Obtained by multiplying the figure in the previous column by a factor which is calculated from values obtained with mixtures of the untreated urine and known amounts of Bayer 205. This factor is usually between 1.10 and 1.25.

All the figures in the above Table represent mg./100 ml.

of course, differentiate between Bayer 205 and similar substances which might be produced from this drug in the animal body. The use of this ultrafiltration method for urine probably ensures, however, that relatively simple substances produced from Bayer 205 will not be estimated with the parent substance since the membranes used are completely permeable to *m*-aminobenzoic acid. *m*-Amino-*p*-toluic acid and  $\alpha$ -naphthylamine-4:6:8-trisulphonic acid, the other amines which, according to Lang [1931], are produced by acid hydrolysis of Bayer 205, are retained by the membranes to a slight extent only.

*Retention of Bayer 205 in the plasma following injection of the drug*

A preliminary investigation to determine the length of time during which injected Bayer 205 could be detected in the plasma of rabbits following a single or a series of injections of the drug was described in an earlier paper [Dangerfield *et al.* 1938]. The results obtained suggested that a series of injections of small amounts results in a more satisfactory maintenance of a significant Bayer 205 level (above 1 mg./100 ml. of plasma) than does a single injection of a much larger amount. Since this problem is of considerable interest to those who are concerned with the prophylactic use of the drug in tropical countries, it was decided to extend these observations in order to obtain continuous curves to show the amount of Bayer 205 in the plasma of rabbits following (a) a large dose of the drug (equivalent to 6.3 g. for a 70 kg. man), and (b) three injections of one-third this amount, the three injections being made at intervals of about 5 weeks.

*Experimental details.* Three rabbits (group A) each received an intravenous injection of 0.09 g. Bayer 205/kg., and three others (group B) received 0.03 g./kg. Further injections, each of 0.03 g./kg., were given to the second group 36 days and 73 days after the first injection.

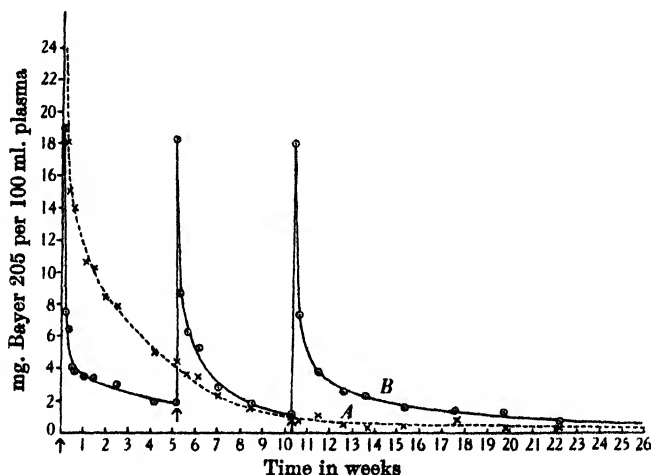


Fig. 1. Bayer 205 in the plasma of rabbits following the injection of the drug.  $\times$  - - -  $\times$  Group A (one injection).  $\circ$  - -  $\circ$  Group B (three injections). The arrows indicate when the injections were made.

Blood samples (about 6 ml.) were withdrawn from the marginal vein of the ear before, and 3 and 24 hr. after, each injection and at suitable intervals during the remaining periods. The blood was oxalated and centrifuged immediately. The amount of Bayer 205 in the plasma was then determined as previously

described [Dangerfield *et al.* 1938]. The determinations were made with a colorimeter and in most cases by the comparator method described in this paper; no significant difference between the two results was observed. Determinations of the "blank value" for normal rabbits were made at frequent intervals. These proved to be practically constant at the equivalent of approximately 1 mg. of Bayer 205/100 ml. of plasma and all values for the experimental animals have been corrected accordingly.

The results of this experiment are recorded in Fig. 1, the values recorded representing averages for the two groups of rabbits.

### DISCUSSION

Further study has shown that the previously described method of determination of Bayer 205 (or Antrypol) in plasma [Dangerfield *et al.* 1938] presents few difficulties and is satisfactory for the determination of amounts of the drug which are likely to be significant. No serious modification of the method has been found necessary, but the use of a Lovibond comparator with appropriate discs renders the determination much easier and makes it possible for measurements to be made when a colorimeter is not available. Throughout the whole of this work the authors have kept in mind the probability that workers in tropical countries, carrying out investigations under somewhat difficult conditions, might wish to make Bayer 205 determinations. For this reason, the observation made here, that serum and plasma give the same values, will be of special interest. Further experiments are now being made to determine the best method of preserving serum which will allow accurate determinations to be made several days or even weeks after the blood has been collected.

A considerable amount of time has been devoted to rendering the method applicable to urine, for such determinations will have to be made before a true picture of the behaviour of Bayer 205 in the body can be obtained. Although there is little or no chemical evidence that the drug is excreted in the urine after its injection into an animal, there is no doubt that this excretion does occur. The investigations on man now being carried out, involving an attempt to trace the fate of most of the Bayer 205 injected, necessitate measurement of the excretion, and the method described in this paper appears to be quite satisfactory for this purpose. The method is, unfortunately, more complicated than that for plasma or serum, but there is as yet no indication that urinary Bayer 205 determinations on patients receiving this drug will normally be needed. The more important value, as far as treatment is concerned, is almost certainly the plasma Bayer 205 level. If it is found necessary to make determinations on urine, the latter can be preserved by the addition of toluene, and the hydrolyses and determinations made at a later date.

The results of the experiment on rabbits confirm the suggestion made previously that for the maintenance of a significant level of Bayer 205 in the plasma it is better to have a series of injections rather than one massive dose (cf. Fig. 1). No evidence has yet been obtained that the latter builds up a higher reserve in the body, and from other evidence available it seems probable that a considerable part of the extra Bayer 205 injected in the large dose is excreted in the urine during the first few days. The amount of Bayer 205 or Antrypol usually injected into man for the curing or prevention of sleeping sickness varies very considerably [cf. Findlay, 1930, pp. 262-8], but the customary dose is 1 or 2 g. for the average man. These injections are often repeated at intervals of a few days or every week, and the total amount injected over a period of 6 months may be 5 g. or even as much as 7-10 g.

The long retention of Bayer 205 in the animal body is remarkable. It seems probable that the persistence of Bayer 205 in the plasma and tissues accounts for the very satisfactory prophylactic value of this drug as far as sleeping sickness is concerned. Information which would be very useful in this connexion can perhaps be classified as follows: (i) the minimum plasma-Bayer 205 level which will normally protect against sleeping sickness when the individual is exposed to infected tsetse flies, (ii) the best method of ensuring that this level will be exceeded without excessive administration of the drug, and (iii) the simplest way of making Bayer 205 determinations on the plasma of patients. The method described in this paper is suitable for the last-named determinations in ordinary field laboratories, but the other questions cannot be dealt with so readily. The minimum "protective" Bayer 205 level for man can best be established by numerous determinations on patients who develop the disease after treatment with the drug. In that way it may be possible to say that if the amount of Bayer 205 in the plasma falls below a certain value the protection against trypanosomiasis is not adequate. Another method, and the one which the authors propose to adopt, involves experiments on small animals (rabbits, mice etc.), since the natural conditions (man as the experimental animal and infection by the bite of infected tsetse) cannot be reproduced readily in this country.

The best method of maintaining a protective level of Bayer 205 in the plasma can only be satisfactorily established when the average minimum protective level has been fixed, but for present purposes it might be assumed (very tentatively) that values above 1 or 1.5 mg./100 ml. of plasma may be significant under normal conditions. If this is not so, the protective effect observed in investigations on volunteers injected with Bayer 205 and subsequently exposed to the bites of infected tsetse flies [Duke, 1934] and the almost universal agreement that small amounts of the drug will give protection for several weeks [cf. review of literature by Findlay, 1930] are difficult to explain; otherwise it would be necessary to assume that the protective action is not directly related to the Bayer 205 present in the plasma and in tissue fluid. In the light of these chemical studies, therefore, there does not appear to be justification for either a very large dose of the drug (greater than 2 g. for an average man) or for several injections at very short intervals (e.g. every few days). A series of three injections made every 4 or 5 weeks should serve to maintain the plasma-Bayer 205 level above 1 mg./100 ml. for a total period of 4 or 5 months. It is not suggested however that the valuable clinical observations of those who prefer to give several injections over a period of 1 or 2 weeks should be disregarded, for until a "minimum protective level" (if such a value does exist) can be determined, it would be undesirable to contrast critically the various methods of prophylactic treatment.

The possibility that Bayer 205 is stored in certain parts of the body is being studied at the present time. These experiments are as yet incomplete, but there seems to be no doubt that the drug is not stored to any significant extent in organs such as the liver, spleen etc. Other experiments, which will be described in a later paper, show that some type of combination occurs between Bayer 205 and plasma proteins, and the long retention of the drug in the animal body is most probably due to its combination with plasma and tissue proteins.

#### SUMMARY

1. Further observations have been made on the method of determination of Bayer 205 (or Antrypol) previously described [Dangerfield *et al.* 1938]. The technique has been simplified by the preparation of coloured discs for a Lovibond comparator.

2. Plasma and serum obtained from a Bayer 205-containing blood contain the same amount of the drug.

3. A modification of the method has been devised for the determination of small amounts of Bayer 205 in urine. The difference between the values for the urine and an ultrafiltrate obtained by filtration through a standard collodion membrane is a measure of the Bayer 205 present. This difference gives a minimum value which can be rendered more accurate by the use of a correction value. This factor is usually between 1.10 and 1.25, and it can be determined quite easily.

4. Investigations have been carried out which support the suggestion made in a previous paper that a course of injections of the drug results in a more satisfactory maintenance of a significant amount of Bayer 205 (or Antrypol) in the plasma over a long period than does a single injection of the same total amount.

5. Some possible reasons for the long retention of the drug in the plasma are tentatively discussed.

The authors would like to express their gratitude to the Medical Research Council for personal grants to two of them (W. G. D. and later J. C. B.), and for a grant which has, in part, covered the expenses of this investigation. To Mr G. S. Fawcett of "The Tintometer Ltd." we are indebted for help with the preparation of coloured discs for the Lovibond comparator, and thanks are tendered to Messrs B.D.H. for a supply of Antrypol.

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## XII. EXPERIMENTS ON THE METABOLISM OF GLYCINE

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THE metabolism of glycine has been a subject of controversy for a long time. As the simplest amino-acid, it was chosen as one of the first objects of investigation. But in the course of research it was seen that glycine behaved differently in many ways from the higher members of the series and that biochemical and physiological effects easily demonstrated with the higher aliphatic amino-acids proved to be absent or inconsistent with glycine.

The deamination of an amino-acid is considered to be one of its chief metabolic changes, and it was this mechanism on which early research was carried out. As long as the latter was confined to experiments involving the metabolism of the whole body, the results obtained by different investigators seemed to agree in general that glycine when fed *per os* [Salkowski, 1880; Nencki & Schultzen, 1872; Friedmann, 1908; Ringer & Lusk, 1910] or injected into the animal was almost quantitatively deaminated, the equivalent amount of  $\text{NH}_3$  or urea being excreted by the animal. More recent experiments of Kiech & Luck [1931], however, demonstrated the existence of a lag between amino-N disappearance as estimated from the N content of the minced carcass, and urea formation following glycine administration. They conclude that "calculations of the extent of amino-acid metabolism from the amount of urea excreted during the first 8 hr. following amino-acid administration are likely to be seriously incorrect". Contradictions also arose when attempts were made to localize the deaminating effect on glycine by perfusion experiments with liver. Salaskin [1898] was the first to report urea formation during perfusion of liver with blood containing added glycine. Fiske & Karsner [1914], who repeated the experiment with improved technique, could not confirm these findings, whereas Jansen [1915] obtained small amounts of urea after perfusion. It is conceivable that the long duration of the experiments used by some of these workers (4-5 hr.) may finally have caused decomposition of the urea. The fate of the 2-C skeleton remaining after the deamination of glycine is also controversial. Feeding experiments such as carried out by Ringer & Lusk [1910] on phloridzinized dogs demonstrated an increased carbohydrate excretion following glycine administration which Pflüger [1910] could not confirm. More recently the same negative result was reported by Wilson & Lewis [1930], whereas Butts & Dunn [1935] observed no glucogenetic effect in one case, but report a small increase in carbohydrate content in another case.

Nor has agreement been reached hitherto either by means of the slice technique or by the use of tissue extracts. After Neubauer [1909] had discovered the principle of oxidative deamination in phenyl-substituted amino-acids. Patey & Holmes [1929] observed  $\text{NH}_3$  production in chopped kidney tissue with added glycine. Krebs [1933] observed a slight deamination of glycine following its incubation with kidney and liver slices, but practically no effect was observed with kidney extracts, although a great number of other amino-acids were readily deaminated. Later Krebs [1935, 1], Bernheim & Bernheim [1935] and Kisch [1936],

confirmed the stability of glycine to deamination and oxidation, and Neber [1936] showed that of all the amino-acids tested glycine alone resisted deamination by intestinal mucous membrane. Reid [1936] obtained an increase in liver glycogen after injection with alanine, but a decrease following injection of glycine. Finally, similar results were experienced by Bach & Holmes [1937] when investigating the glucogenetic effect of amino-acids following their incubation with liver slices. Glycine proved to be the only amino-acid tested which caused no formation of carbohydrate, in fact a carbohydrate disappearance.

Thus it appears that glycine is probably metabolized by the body when fed to the whole organism, but the experimental conditions under which its deamination might consistently be demonstrated *in vitro* in different organs are not fully known. An attempt to elucidate such conditions and to clarify the mechanism of deamination seemed to be justified and represented the problem for this work.

### METHODS

*Animals used.* Young rats, chiefly albinos, all males with an average wt. of 200 g. normally fed on ordinary stock laboratory diet, were used for all experiments with surviving tissue slices and chopped organs. Cats were used for the perfusion experiments. Tissue extracts were prepared from organs of pig.

*Method of perfusion.* Two cats were used for each experiment, and ether was used as anaesthetic. One cat served as blood donor; from it about 140 ml. blood were drawn to which about 80 ml. blood of the second cat were added later. The blood was defibrinated, filtered and made up to 400 ml. with bicarbonate-Ringer solution. In the meantime a cannula was inserted into the hepatic portal vein of the liver of the second cat. When the kidney was perfused the cannula was inserted into the renal artery. The gall bladder was ligated in the case of liver perfusion. The respective organs were then removed from the animals, the blood of the second animal was treated in the same way as that of the first and added to the blood-Ringer solution. The Dale & Schuster apparatus was used for the perfusion with a flask oxygenator. Care was taken that the oxygenation was efficient. The blood colour changed from dark red after it had passed the organ to bright red after oxygenation. The speed of perfusion was such that the whole volume of the perfusion fluid circulated in the system within 7 min. Samples of the organs were clipped off at the beginning and at the end of the experiment and the cut surface was seared with a hot iron. The temp. was kept at 37° and it was observed that the perfusion fluid was evenly distributed throughout the organ perfused. The perfusion fluid circulated through the organ for 20 min. in absence of substrate. After that time glycine solution was added. Samples were taken at suitable intervals, the volume of each being recorded in order to control the volume of the remaining fluid. The duration of the perfusion varied in the different experiments from 90 to 150 min. At the end of the experiment the remaining perfusion fluid was emptied from the apparatus. The samples were immediately deproteinized by means of trichloroacetic acid, and  $\text{NH}_3$ - and  $\text{NH}_2$ -N estimations were carried out at once. For the isolation of glycine,  $\text{HgCl}_2 + \text{HCl}$  was used as deproteinizing agent; the procedure of isolation is described below.

*Experiments on surviving tissue slices and extracts.* Technique of slicing was the same as described previously [Bach & Holmes, 1937]. The wt. of tissue slices was 100 mg. wet wt. in all exp. Slices could be conveniently cut from kidney, liver and brain. It was more difficult in the case of spleen, but it was possible to obtain sufficiently thin slices from this organ after some practice. The spleen slices then showed satisfactory respiration. The blood attached to the spleen



slices was not removed. Portions of the thinner parts of the diaphragm were used. Testis material was used without further treatment. The mucous membrane of the small intestine was used after washing the latter with water, cutting it lengthwise and scraping it off the intestinal wall. For the experiments with frog muscle an extract was prepared in the usual way. Heart tissue was cut in slices with scissors as it was not possible to slice it in the ordinary way with the razor. The tissue was in all experiments suspended in 3 ml. phosphate buffer pH 7.2, this volume including any added substrates.

Kidney extract was made from acetone powder [Keilin & Hartree, 1936] by suspending it in buffer solution, shaking it for 10 min. at room temperature followed by centrifuging. 2 ml. supernatant fluid, corresponding to 100 mg. acetone powder, were used for the experiment, 1 ml. buffer or substrate being added.

Barcroft cups and manometers were used. In the aerobic experiments the cups were evacuated and filled with  $O_2$ . In the anaerobic experiments the cups were 3 times evacuated and filled with  $N_2$  passed over hot Cu filings. The exp. temp. was  $38^\circ$ .

The substrates after adjustment to pH 7.2 were in most cases added in dangling Keilin cups which were shaken off after equilibrium had been obtained. In the centre pot of the Barcroft cups was placed filter paper saturated with 0.3 ml. of 10 % KOH.

At the end of the experiment 2 ml. were drawn from the cups and made up to 10 ml. Aliquot portions of this fluid were used for  $NH_3$  and urea estimations. Other portions used for estimations of total, amino-, amide- and purine-N, creatine and pyruvic acid, were deproteinized either with trichloroacetic acid (final concentration 6 %) or, in the later experiments with dialysed  $Fe(OH)_3$  which gave more consistent results owing possibly to the avoidance of decomposition of the glycine condensation products.

*Estimation of  $NH_3$  and urea.* To the portion reserved for the  $NH_3$  estimation 3 drops 3 % acetic acid were added, and  $NH_3$  estimated after distillation [Parnas & Heller, 1924], collection in 0.01 N HCl and titration with 0.01 N NaOH. The portion of the fluid reserved for the urea estimation was incubated for 30 min. with urease at  $60^\circ$  followed by  $NH_3$  estimation.

*Estimation of  $NH_2-N$ .* The deproteinized fluid was centrifuged, carefully neutralized and made up to 10 ml. To a suitable amount about 10 drops of  $Mg(OH)_2$  suspension were added, the mixture placed on the boiling water bath for 25 min., the fluid being then acidified with 2 drops of glacial acetic acid. The estimation of  $NH_2-N$  was made with the Van Slyke apparatus.

*Estimation of amide-N.* To an aliquot portion of the deproteinized fluid  $H_2SO_4$  was added to a final concentration of 5 %. It was placed on a boiling water bath for 30 min. after which it was neutralized and the  $NH_3$  estimated.

*Estimation of total N.* Total N was estimated in a Kjeldahl apparatus, conc.  $NH_3$ -free  $H_2SO_4$  with added Se and Cu salts being used for the incineration. The titration was carried out with 0.01 N HCl.

*Estimation of purine-N.* To 2 ml. deproteinized fluid 2 ml. 40 %  $NaHSO_3$  and 2 ml. 10 %  $CuSO_4$  were added. The reddish precipitate turned brown after 3 min. boiling. The fluid was centrifuged, the precipitate washed with 1 % acetic acid and centrifuged again followed by total N estimation on the precipitate [Krüger & Schmidt, 1905; Schmidt, 1933].

*Estimation of creatine.* A modification of Walpole's direct method [Walpole, 1911] of estimating creatine was used, diacetyl serving as reagent; it was prepared by distillation of dimethylglyoxime with  $H_2SO_4$  [Walpole, 1911]. To 5 ml. experimental solution 0.5 ml. sat.  $Na_2CO_3$  and 2 drops diacetyl were added.

The mixture was immediately placed in a boiling water bath for exactly 1 min. and then in cold water for 5 min. After a further period of 20 min. during which the samples were left standing at room temperature the red colour was measured as described below. Standard solutions with known amounts of creatine and controls with water were prepared in the same way. Since the control samples with water yielded a yellowish tint with the reagents alone, methods had to be developed to eliminate this interfering colour. Neither a colorimeter nor a tintometer served the purpose. In the later experiments the readings were carried out by means of a spectrophotometer. The light passing through the coloured solution of the water control was compared at different wave-lengths with that

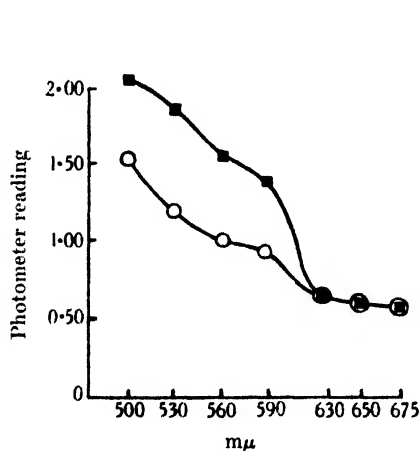


Fig. 1.

Fig. 1. Photometer readings. ■—■ With 100 g. creatine solution.  
○—○ With the water control.

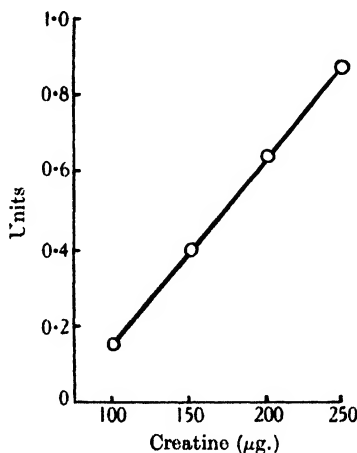


Fig. 2.

Fig. 2. Standard curve for creatine estimation. For definition of unit see text.

transmitted by known amounts of creatine. It will be seen from Fig. 1 that the two curves are almost contiguous down to 630 mμ (absorption =  $b$  arbitr. units), below which they show a divergence reaching a maximum value at approximately 530 mμ (absorption =  $a$  arbitr. units). The final standardizing curve was plotted by estimating  $a$  and  $b$  both for the water control and for mixtures containing different known amounts of creatine ( $a'$  and  $b'$ ). A straight line was obtained when values of creatine in arbitrary absorption units from the equation:  $(a' - b')_{\text{creatin}}$  minus  $(a - b)_{\text{control}}$  were plotted against μg. creatine (Fig. 2). The influence of glycine on the colour developed during the creatine estimations was found to be negligible. That of glycocyamine was allowed for by adding this substance to the water control.

**Estimation of pyruvic acid.** The estimation of pyruvic acid was carried out on the deproteinized fluid according to the method of Clift & Cook [1932]. Special care was taken for reasons described that the excess  $\text{NaHSO}_3$  was titrated with  $\text{I}_2$  immediately in order to avoid possible decomposition of the product of condensation of glycine with pyruvate (see below).

**Reagents.** I have to thank Dr D. E. Green for a gift of coenzyme solution and Mr E. M. Crook for that of coenzyme II. The other reagents were obtained from British Drug Houses, Ltd.

## EXPERIMENTAL

*In vitro* experiments were first made on the deamination of glycine and *dl*-alanine under conditions in which many amino-acids besides alanine are readily deaminated. In all the cases in which  $\text{NH}_3\text{-N}$  was estimated, urea-N was determined at the same time, the total of both being given as " $\text{NH}_3\text{-N}$ ". The results from Table I show clearly that while alanine readily gives rise to  $\text{NH}_3$  production, no such formation of  $\text{NH}_3$  takes place under the same experimental conditions with glycine.

Table I.  $\text{NH}_3$  formation from glycine and *dl*-alanine in extracts of pig kidney

Experimental period: 2 hr.					
Kidney powder used for extract mg.	Amino-acid-N added mg.	mg. $\text{NH}_3\text{-N}$			Extra $\text{NH}_3\text{-N}$ formation as % of added $\text{NH}_2\text{-N}$
		Without added substrate	With added amino-acid	Extra $\text{NH}_3\text{-N}$ formation	
Glycine					
100	0.56	0.14	0.16	0.02	0.4
200	1.12	0.21	0.12	-0.09	-8
100	1.12	0.05	0.05	0	0
100	1.12	0.08	0	-0.08	-7
50	0.56	0.07	0.04	-0.03	-5
250	1.12	0.14	0.14	0	0
*	1.12	0.08	0.08	0	0
Alanine					
100	0.47	0.14	0.294	0.154	65†
200	0.94	0.21	0.30	0.18	38†
100	0.94	0.05	0.35	0.30	64†
100	0.94	0.08	0.35	0.27	57†
50	0.47	0.07	0.24	0.19	81†
250	0.94	0.14	0.42	0.28	60†
*	0.94	0.08	0.40	0.32	68†
Average for alanine					62

\* 1 g. rat kidney was ground with sand and buffer and centrifuged, the supernatant fluid being taken for the experiment.

† % disappearance is calculated from the amount of added *d*-alanine (i.e.  $\frac{1}{2}$  *dl*-alanine).

The same difference in behaviour between the two amino-acids was observed regarding the  $\text{O}_2$  consumption. Fig. 3 gives the course of the respiration of kidney extract in presence of glycine + *dl*-alanine. It will be seen that while with alanine  $\text{O}_2$  consumption reached the theoretical value calculated on the basis of oxidative deamination of the *d*-isomeride, glycine does not increase the  $\text{O}_2$  consumption of the tissue at all. It is also shown that an addition of glycine + alanine does not affect the respiration as compared with that of alanine alone in tissue extracts. This phenomenon is equally shown by comparison of columns 4 and 6 in Table II in which the results of further respiration experiments are recorded. A comparison of columns 3 and 5 reveals a slight inhibition of  $\text{O}_2$  consumption in presence of glycine alone.

Table III shows the effect of a mixture of glycine and alanine when incubated with various tissues. The object of these experiments was to investigate whether a "Stickland effect" such as observed in bacteria [Stickland, 1934; Woods, 1936] would affect  $\text{NH}_3$  production. Results from Table III show that this is not the case. There is very little or no difference between the formation of  $\text{NH}_3$ , the disappearance of  $\text{NH}_2\text{-N}$  and the  $\text{O}_2$  consumption in presence of alanine alone and those in presence of alanine plus glycine.

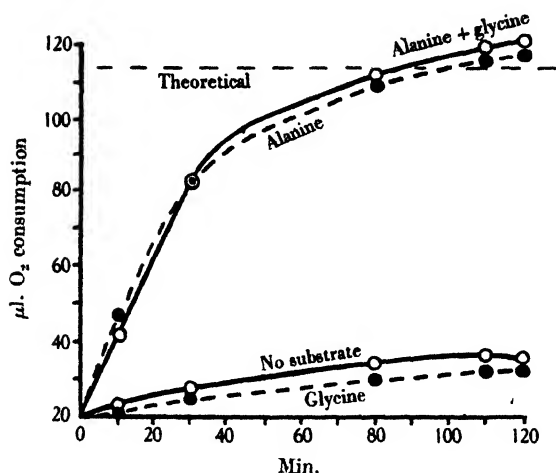


Fig. 3. Respiration of pig kidney extract with added *dl*-alanine and glycine.

Table II. *Respiration of kidney extract in presence of glycine and dl-alanine*

Duration of exp. (min.)	Amino-acid conc. (%)	μl. O <sub>2</sub> consumption			
		Without added substrate	With alanine	With glycine	With glycine and alanine
60	0.1	25	169	15	174
60	0.2	87	322	23	—
60	0.2	—	322	10	322
90	0.1	31	185	21	192
90	0.2	93	387	28	—
90	0.2	—	358	19	355

Table III. *Experiments with various tissues in presence of a mixture of glycine and dl-alanine*

Duration of exp. min.	Tissue	Substrate	Amino-acid-N added mg.	With added substrate		
				Extra NH <sub>3</sub> -N formed mg.	Extra O <sub>2</sub> consumption μl.	% of NH <sub>2</sub> -N disappeared mg.
60	Kidney extract	Glycine	0.56	0.03	—	—
60		<i>dl</i> -Alanine	0.47	0.26	144	—
60		<i>dl</i> -Alanine + glycine	1.03	0.33	149	—
100	Kidney extract	Glycine	1.12	—	21	0
100		<i>dl</i> -Alanine	0.94	—	399	23
100		<i>dl</i> -Alanine + glycine	2.06	—	377	31
60	Kidney extract	Glycine	1.12	0.06	10	—
60		<i>dl</i> -Alanine	0.94	0.18	322	—
60		<i>dl</i> -Alanine + glycine	2.06	0.20	322	—
65	Kidney extract and spleen slices	Glycine	1.12	0	35	—
65		<i>dl</i> -Alanine	0.47	0.13	102	—
65		<i>dl</i> -Alanine + glycine	1.59	0.14	97	—

For further evidence determinations of NH<sub>2</sub>-N were carried out, the results of which, shown in Table IV, seem to confirm the previous findings. While in the case of the alanine experiments an average of 82 % of the NH<sub>2</sub>-N (calculated on

Table IV. *Disappearance of NH<sub>2</sub>-N from glycine and dl-alanine in kidney extract*

Experimental period: 2 hr.

NH <sub>2</sub> -N added mg.	Without substrate	mg. NH <sub>2</sub> -N			Disappearance of NH <sub>2</sub> -N in % of NH <sub>2</sub> -N added
		With amino- acid added	Increase with amino-acid	Disappearance of added NH <sub>2</sub> -N	
Glycine					
1.12	0.47	1.50	1.03	0.09	8
1.12	0.47	1.82	1.35	- 0.23	0
1.12	0.80	1.83	1.03	0.09	8
dl-Alanine					
0.94	0.26	0.69	0.43	0.51	108*
0.47	0.26	0.50	0.24	0.23	100*
0.94	0.80	1.24	0.44	0.50	100*
0.94	0.47	1.14	0.67	0.27	58*
0.94	0.47	1.20	0.73	0.21	45*
Average for alanine					82*

\* Disappearance in % of added *d*-alanine-N (i.e.  $\frac{1}{2}$  *dl*-alanine).

the basis of the *d*-isomer) disappeared, practically all the glycine NH<sub>2</sub>-N was present in most of the cases at the end of the experimental period. Another phenomenon observed was a discrepancy between the average disappearance of alanine-N of 82 % and an NH<sub>3</sub>-N formation of 62 % (according to Table I). (Both series of determinations were carried out in the same experiment in order to obtain comparable values.) This phenomenon has previously been observed by Krebs [1933] and also by Neber [1936].

Table V. *NH<sub>3</sub> formation and O<sub>2</sub> consumption in tissue slices of various organs in presence of glycine*

Tissue wet wt. 100 mg. Duration 2 hr. Amount of glycine added 1.12 mg. glycine-N, except in exp. \* in which it was 0.96 mg. Glycine conc.: 0.2 % in all experiments.

Tissue	Extra NH <sub>3</sub> -N with added glycine mg.	Glycine-N disappeared mg.	Extra O <sub>2</sub> con- sumption with added glycine, μl. after 60 min.
Rat kidney	0.04	—	- 40
Rat heart	0.03	—	—
Rat heart	0	0.05	- 9
Rat spleen	0.05	—	- 16
Rat spleen	- 0.03	—	- 12
Rat spleen	0.01	—	—
Rat intestine	0.01	—	—
Frog muscle	- 0.04	—	0
Rat kidney	0.03	- 0.10	- 12
Rat heart	0.07	0.11	—
Rat kidney*	—	0.16	—
Rat kidney* 3.7 mg. glutathione	—	0.09	—
Rat kidney pH 7.8	—	0.07	—
Rat kidney pH 6.8	—	0.09	—
Rat testis	—	0	—
Rat brain	—	0.15	—
Rat spleen	—	0.12	—
Rat kidney	—	—	- 61
Rat kidney	—	—	- 5
Pig kidney	—	—	- 5
Rat liver	—	—	14
Rat diaphragm	—	—	6

In the experiments recorded in Table V tissue extracts were replaced by tissue slices of various organs. None of the tissues used produced any appreciable amount of  $\text{NH}_3$  or urea from glycine and none or little  $\text{NH}_2\text{-N}$  disappeared. In fact the respiration experiments revealed a slight but distinct inhibition in presence of glycine in almost all cases.

From the results obtained so far it seemed obvious that the deamination of glycine cannot be demonstrated by incubating it *in vitro* with either tissue extract or tissue slices under conditions such that with other amino-acids  $\text{NH}_3$  or urea is readily formed and  $\text{O}_2$  is taken up. Therefore perfusion experiments with added glycine on the intact organ were carried out as recorded in Tables VI and VII. The technique has been described earlier.  $\text{NH}_3\text{-}$  and  $\text{NH}_2\text{-N}$  estimations

Table VI. *Perfusion of cat liver*

Quantities: mg. N/100 ml. perfusion fluid or per g. liver.

Time course of perfusion I min.	NH <sub>3</sub> - and urea-N						Amount of glycine- N added (mg.)
	In perfusion fluid			In perfused liver			
	NH <sub>3</sub> -N	Urea-N	NH <sub>3</sub> - plus urea-N	NH <sub>3</sub> -N	Urea-N	NH <sub>3</sub> - plus urea-N	
	Without added glycine						
0	0.4	4.6	5.0	0.18	0	0.18	0
20	0.4	4.1	4.5	0.18	0	0.18	0
	With added glycine						
25	0.28	5.57	5.85	—	—	—	55
80	0.63	8.17	8.80	—	—	—	55
110	1.12	9.28	10.40	0.12	0.35	0.47	55
Final result perfusion I	+ 0.72	+ 5.18	+ 5.90	- 0.06	+ 0.35	+ 0.29	55
Final result perfusion II	+ 3.03	+ 1.33	+ 5.36	0	- 0.38	- 0.38	96.5
Final result perfusion III	0.14	+ 3.68	+ 3.82	- 0.03	- 0.18	- 0.21	33.6

Table VII. *Perfusion of cat kidney*

Quantities: mg. N/100 ml. perfusion fluid or per g. kidney.

Duration of perfusion min.	NH <sub>3</sub> and urea-N <sub>2</sub>				NH <sub>2</sub> -N in perfusion fluid
	In perfusion fluid			In perfused kidney NH <sub>3</sub> -plus urea-N	
	NH <sub>3</sub> -N	Urea-N	NH <sub>3</sub> -plus urea-N		
	Without added glycine				
0	0.28	7.00	7.28	0.71	10.5
15	0.28	—	—	—	7.35
	With added glycine				
75	0.70	—	—	—	—
105	0.28	5.60	5.88	0.77	43.7
Final result	0	- 1.40	- 1.40	+ 0.06	36.35
	Amount of glycine-N added				34.3

were performed both on the perfusion fluid and the organs used. Glycine was isolated from the former as the  $\beta$ -naphthalene-sulphonic compound.

In Table VI the course of a liver perfusion (I) has been briefly outlined whereas from liver perfusions (II) and (III) only the final balance is given for

simplicity's sake. Table VII shows the course of kidney perfusion and also includes  $\text{NH}_2\text{-N}$  determinations.

From the final results of Tables VI and VII it is evident that the  $\text{NH}_3$  and urea formation observed during the perfusion is insignificant, representing only 5–10 % of added glycine-N. The results of the perfusion without added substrate revealed no significant changes during the initial period. The changes in the N content of the perfused organs were negligible. The  $\text{NH}_2\text{-N}$  content in the final perfusion fluid corresponded approximately to the amount of glycine-N added at the beginning of the experiment (34.3 % added as glycine-N and 36.3 % found as  $\text{NH}_2\text{-N}$  at the end of the experimental period). The method of  $\text{NH}_2\text{-N}$  determination was checked by adding a known amount of glycine to a quantity of perfusion fluid, the  $\text{NH}_2\text{-N}$  of which had been estimated while in circulation. The estimated  $\text{NH}_2\text{-N}$  values were found to agree satisfactorily with the calculated amounts, the average experimental error being 6 %. By the isolation of glycine from the perfusion fluid it was ascertained that the  $\text{NH}_2\text{-N}$  found at the end of the experiment was in fact glycine-N. This was done by adding to 250 ml. of the fluid, 100 ml. 2 N HCl and 100 ml. 5 %  $\text{HgCl}_2$ , filtering the precipitate, removing the Hg by  $\text{H}_2\text{S}$ , neutralizing and finally concentrating *in vacuo* to c. 20 ml. A small precipitate filtered off was disregarded. To the clear filtrate an ethereal solution of 3 g.  $\beta$ -naphthalenesulphochloride was added and the mixture was shaken for 6 hr. during which 24 ml. of N NaOH were gradually added. The mixture was again filtered and acidified with 2 N HCl, the precipitate filtered and recrystallized from water. The yield varied in the different experiments from 38 to 62 % of the amount expected in accordance with the  $\text{NH}_2\text{-N}$  determinations. The substance was  $\beta$ -naphthalenesulphoglycine. (Found: C, 54.08; H, 4.39; N, 5.53; S, 11.9. Calc. for  $\text{C}_{12}\text{H}_{11}\text{O}_4\text{NS}$ : C, 54.3; H, 4.16; N, 5.28; S, 12.07.) No other  $\text{NH}_2$  compound was found to be present in the perfusion fluid.

Further experiments (not recorded) were made to investigate the effect of fixatives on the deamination of glycine. In liver slices no  $\text{NH}_3$  formation or disappearance of  $\text{NH}_2\text{-N}$  occurred, however, with glycine in presence of fixatives. Ornithine was added as fixative for  $\text{NH}_3$  and  $\text{NaHSO}_3$  for glyoxylic acid, possibly formed. The presence of glucose also had no effect when added with glycine to kidney slices.

Further, the possibility of a collaboration of more than one organ in the metabolism of glycine was considered; since as mentioned earlier glycine administered *per os* appears to be readily attacked. The example of the formation of uric acid by collaboration of liver and kidney, as shown by Schuler & Reindel [1933], inspired these investigations. Mixtures of kidney and liver tissues and of kidney and spleen tissues were chosen.

In the experiments in which glycine was added to such mixtures (Table VIII) it will be seen, in the case of kidney slices plus spleen slices, that while no  $\text{NH}_3$  or urea was formed,  $\text{NH}_2\text{-N}$  disappeared in some cases to an extent of approximately 70–95 % of the  $\text{NH}_2\text{-N}$  added. No disappearance of  $\text{NH}_2\text{-N}$  however was observed in the case of the mixture of kidney and liver slices, irrespective of the addition of substrates such as alloxan or ketoglutaric acid. (Alloxan is known to catalyse the oxidation of amino-acids *in vitro* [Strecker, 1862] and  $\alpha$ -ketoglutaric acid has been reported to serve as an "acceptor" for amino groups [Braunstein & Kritzman, 1937, 1].) Kidney slices and kidney extract produced equal effects when mixed with the spleen slices.

Estimations of total N were carried out on the deproteinized filtrates of the experimental fluid. An example is given in Table VIII. No change of total N was observed, hence the  $\text{NH}_2\text{-N}$  disappearing was not used for synthesis of proteins.

Table VIII. *N* changes and  $O_2$  consumption in mixtures of tissue slices in presence of glycine

Duration of exp.: 2 hr. Amount of glycine-N added: 1.12 mg. except in exp. † where 0.96 mg. was used. Rats were used for all tissue slices. Concentration of kidney extract: 50 mg. dried powder from pig kidney.

Mixture of tissue used	Extra $NH_3$ -N with added glycine mg.	mg. $NH_2$ -N				Extra $O_2$ consumption with added alanine, $\mu$ l. after 60 min.	% of glycine-N disappeared
		Without glycine	With glycine	Difference	Disappeared		
Kidney slices† + Liver slices	—	0.20	1.07	0.87	0.09	—	9
Kidney slices† + Liver slices + Alloxan <i>M</i> /100	- 0.02	0.17	1.14	0.97	0	- 2	0
Kidney slices + Liver slices + Ketoglutaric-acid <i>M</i> /40	- 0.01	0.17	1.22	1.05	0.07	- 38	7
Kidney slices + Spleen slices	0.04	0.38	0.63	0.25	0.87	49	78
Kidney slices + Spleen slices	0.02	—	—	—	—	—	—
Kidney slices + Spleen slices	0.03	0.34	0.61	0.27	0.84	—	75
Kidney extract + Spleen slices	0.10	—	—	—	—	- 12	—
Kidney extract + Spleen slices*	0	0.73	0.80	0.07	1.05	29	94
Kidney extract + Spleen slices	0.01	0.48	0.81	0.33	0.79	—	70

mg. Total N

	Without glycine	With glycine	Difference	Disapp.
Estimation of total N in exp. *	0.06	1.20	1.14	0

The disappearance of  $NH_2$ -N in presence of mixed kidney and spleen tissue, not occurring in presence of either of the tissues alone, may be interpreted either as an enzymic effect or an effect of non-enzymic substances present in the tissue mixture. In order to answer this question, further experiments with *Kochsaft* of various tissues were carried out. The results are shown in Table IX. Kidney *Kochsaft* added to kidney slices caused a disappearance of approximately 25 % of the  $NH_2$ -N. Spleen *Kochsaft* alone had about the same effect, which was hardly increased by addition of kidney *Kochsaft*, but which appeared to be a little intensified in presence of kidney slices. Heart *Kochsaft* caused no effect. A comparison of the results of Table VIII with those of Table IX shows that the effect of *Kochsaft* alone or of spleen *Kochsaft* plus kidney slices on the disappearance of  $NH_2$ -N was considerably smaller than that of kidney slices plus spleen slices.  $O_2$  consumption was slightly inhibited with added glycine, as in previous experiments, and extra  $NH_3$ -N formed in presence of glycine was insignificant.

The partial disappearance of  $NH_2$ -N under the experimental conditions described, which was not accompanied by a corresponding  $NH_3$  or urea formation led to an investigation of other products of decomposition of glycine. The possibility of the formation of amides and creatine was considered next.



Table IX. *Experiments with kidney slices and kidney Kochsaft in presence of glycine*

		mg. $\text{NH}_2\text{-N}$						
Tissue	Substrate other than glycine	Without glycine	With glycine	Difference	Disapp. of $\text{NH}_2\text{-N}$	Disapp. of $\text{NH}_2\text{-N}$ in % of added $\text{NH}_2\text{-N}$	Glycine-N added mg	Other observations
Kidney slices	Kidney <i>Kochsaft</i>	0.22	1.10	0.88	0.27	23	1.15	Extra $\text{O}_2$ with glycine = -46
Kidney slices	Kidney <i>Kochsaft</i>	0.19	1.02	0.83	0.32	28	1.15	Extra $\text{O}_2$ with glycine = -13
Kidney slices	Kidney <i>Kochsaft</i>	0.14	0.96	0.82	0.33	29	1.15	Extra $\text{NH}_3$ 0.15 mg.
Kidney slices	Kidney <i>Kochsaft</i> *	0.32	1.18	0.86	0.29	25	1.15	Extra $\text{NH}_3$ 0.09 mg.
Kidney slices	Heart <i>Kochsaft</i>	0.16	1.27	1.11	0	0.06	1.17	—
Kidney slices	—	0.01	1.23	1.22	0	0	1.12	—
Kidney slices	Kidney <i>Kochsaft</i>	0.16	1.00	0.84	0.28	25	1.12	Creatine test (Tintometer reading) 60% increase with glycine
Kidney slices	Spleen <i>Kochsaft</i>	0.27	1.04	0.77	0.35	31	1.12	—
Kidney <i>Kochsaft</i>	Spleen <i>Kochsaft</i>	0.28	1.14	0.86	0.26	23	1.12	—
—	Spleen <i>Kochsaft</i>	0.14	1.02	0.88	0.24	21	1.12	—

Note. The *Kochsaft* was prepared by boiling 1 g. rat kidney for 7 min. on the water bath and centrifuging 0.3 ml. of the supernatant fluid was used for all exp. except for exp. \* where 0.6 ml. was used.

The formation of amide-N from glutamic acid has been recorded by Krebs [1935, 2]. No appreciable amount of amide-N however was found such as to justify the suggestion that the glycine-N disappeared had been retained as amide-N.

The formation of creatine from glycine has often been claimed, since numerous investigators have proved that patients suffering from the muscular dystrophies excrete less creatine, and since Brand *et al.* [1929; 1931] had found in these cases that following the injection of glycine creatinuria was increased. The same effect was observed after the administration of glycoeyamine [Bodansky, 1936]. This has been isolated from the urine of dystrophic patients following administration of glycine [Weber, 1935]. It is generally assumed that glycoeyamine acts as a precursor to creatine, the former being methylated by glycine to form the latter. Evidence for the conversion of glycoeyamine into creatine in the body has been given by early workers [Jaffe, 1906; Thompson, 1917]. The methylation was assumed to take place in muscles and in kidney [Bodansky, 1936], although Shibuya [1937] failed to show such an effect in *in vitro* experiments with liver slices, and obtained doubtful results when perfusing liver of rabbits. Finally Davenport *et al.* [1938] supplied further evidence for this assumption.

Glycoeyamine was added to mixtures of kidney and liver slices, in order to see whether it was able to cause  $\text{NH}_2\text{-N}$  disappearance as did *Kochsaft*. It will be seen from Table X that in a mixture of kidney and liver slices with added glycine, 46% of the  $\text{NH}_2\text{-N}$  disappeared in presence of glycoeyamine, whereas no appreciable disappearance of  $\text{NH}_2\text{-N}$  occurred when glycine alone was added. It will also be observed that the individual tissues, i.e. liver alone and kidney alone, had no appreciable deaminating effect in presence of glycoeyamine. Thus glycoeyamine showed an effect similar to that of kidney or spleen *Kochsaft*. There is of course no evidence that glycoeyamine is one of the active constituents of spleen *Kochsaft* or kidney *Kochsaft*, although glycoeyamine has been isolated from the urine. The results of the following experiments on creatine formation, however, offer further support for the action of glycoeyamine.

In one of the experiments of Table IX creatine was determined approximately, and it was found to be present in a larger amount in presence of glycine than in its absence. More experiments of this type were made using chopped rat

Table X. *Disappearance of glycine-N in tissue slices in presence of glycocyamine*

Amount of glycine added: 1.12 mg. glycine-N, final concentration 0.2%. Amount of glycocyamine added: 1.5 mg., final concentration 0.05%.

Tissue	Substrate added other than glycine	mg. NH <sub>2</sub> -N			Disappearance of NH <sub>2</sub> -N	Disappearance of NH <sub>2</sub> -N as % of added NH <sub>2</sub> -N
		Without glycine	With glycine	Difference		
Kidney	Glycocyamine	0.1	1.12	1.02	0.1	9
Liver	Glycocyamine	0.1	1.03	0.93	0.19	17
Kidney + liver	Glycocyamine	0.2	0.81	0.61	0.51	46
Kidney + liver	—	0.2	1.07	0.87	0.09*	9
Kidney + spleen	Glycocyamine	0.35	1.12	0.77	0.35	31

\* The amount of glycine-N added in this exp. was 0.96 mg.

Table XI. *Chopped heart of rat in presence of glycine and glycocyamine*

(Concentration of glycocyamine: 0.1%. Amount of glycine added: 1.12 mg. glycine-N)

Substrate added	NH <sub>3</sub> -N mg.	Amide-N mg.	NH <sub>2</sub> -N mg.	NH <sub>2</sub> -N disapp. mg.	Creatine $\mu$ g.	O <sub>2</sub> consumption
None	0.08	—	—	—	800	—
Glycine	0.26	—	—	—	740	154
Glycocyamine	0.19	—	—	—	770	135
Glycine + glycocyamine	0.22	—	—	—	1000	161
None	0.08	0.02	0.08	—	570	93
Glycine	0.15	0.03	0.09	0.11	570	84
Glycine + glycocyamine	0.19	0.08	0.92	0.28	710	98

heart and adding glycocyamine and glycine. The more reliable results of this series, as the technique for creatine estimation had to be improved in the course of this experimental work, are shown in Table XI. It will be seen that no extra creatine is formed with either glycine alone or glycocyamine alone, but small creatine rises were observed where glycine and glycocyamine had simultaneously been added. Furthermore, the disappearance of NH<sub>2</sub>-N was observed to be greatest when both substrates had been added. It will be seen that in these experiments small but definite NH<sub>3</sub> formation has taken place. Again a small increase in O<sub>2</sub> consumption was observed with added glycine + glycocyamine.

These results may point to a possible *in vitro* methylation of glycocyamine by means of glycine, but the difficulties of estimating accurately small amounts of creatine prevent any definite conclusion in this respect.

Amongst substances possibly responsible for the disappearance of NH<sub>2</sub>-N coenzymes had to be considered. The coenzyme responsible for the deamination of the unnatural amino-acids recently discovered by Warburg & Christian [1938] and Straub [1938] exists in sufficient quantities in most of the tissues and tissue extracts used. Hence an effect by adding it could not be expected.

Table XII shows experiments with cozymase and coenzyme II added to glycine under anaerobic conditions. Coenzyme II showed no effect. Cozymase usually caused a disappearance of NH<sub>2</sub>-N of 40–65%, but sometimes much less.

Further experiments were made with pyruvic acid. Table XIII shows the results in kidney extract. They seem to be much more consistent than the experiments in Table XII and reveal that in presence of pyruvate the further

Table XII. *Effects of cozymase and coenzyme II on the disappearance of glycine-N in tissue slices under anaerobic conditions*

Duration of exp.: 2 hr. Amount of glycine added: 1.12 mg. glycine-N.

Tissue	Addition	With added glycine	
		NH <sub>2</sub> -N disappearance mg.	NH <sub>2</sub> -N disappearance as % of added NH <sub>2</sub> -N
Kidney slices	Cozymase	0.74	66
Kidney slices	None	0	0
Kidney slices	Cozymase	0.48	43
Kidney slices	Cozymase	0.71	62.5
Kidney slices	Cozymase	0.12	10
Liver slices	Cozymase	0	0
Kidney slices	Cozymase	0.08	7
Kidney slices	Coenzyme II	0.14	12.5
Kidney slices	Coenzyme II	0.01	0
Liver slices	None	0	0

Table XIII. *Effect of pyruvate on the disappearance of glycine-N in kidney extract under anaerobic conditions*

Duration of exp.: 2 hr. Amount of glycine added: 1.12 mg. glycine-N (1.02 mg. in exp. \*). Concentration of pyruvate: 1%.

Tissue extract	Substrates added	With added glycine	
		NH <sub>2</sub> -N disappearance mg.	NH <sub>2</sub> -N disappearance as % of added NH <sub>2</sub> -N
Kidney extract	{ Cozymase + pyruvate	0.47	42
	{ Cozymase + pyruvate	0.39	35
	{ Pyruvate	0.41	36.5
	{ Pyruvate	0.40	35
	{ Pyruvate	0.61	40.5*
Boiled kidney extract	{ Pyruvate	0.21	21*
	{ Pyruvate	0.24	21
Water control (no tissue extract)	{ Pyruvate	0 (aerobic)	0 (aerobic)
	{ Pyruvate	0.20	18
	{ Pyruvate	0.16	15.5*

addition of cozymase is unnecessary for the disappearance of NH<sub>2</sub>-N. 35–42% glycine-N disappeared in presence of pyruvate and in presence or absence of cozymase. Boiled extract had a small effect compared with unboiled extract. Under aerobic conditions no disappearance of NH<sub>2</sub>-N occurred when tissue extract was replaced by water, whereas, under anaerobic conditions, a small disappearance was observed. This suggested some catalytic effect of the tissue.

In some examples of large disappearance of NH<sub>2</sub>-N, estimations of NH<sub>3</sub> and urea-N, amide-N, total N and purine-N were performed simultaneously. There was no appreciable change observed in any of the N fractions tested, except in NH<sub>2</sub>-N (Table XIV). This meant that the disappearance of NH<sub>2</sub>-N could not be accounted for by the formation of any of the above N compounds, and suggested that the glycine had not lost its N but had condensed with another substrate, using in this way its NH<sub>2</sub> group to form a nitrogenous condensation product.

In estimating the simultaneous disappearance of pyruvic acid with the disappearance of NH<sub>2</sub>-N the decomposition of a possible condensation product by the

Table XIV. *Simultaneous determination of nitrogenous substances in exp. with added glycine and pyruvate under anaerobic conditions*

Biological material: kidney extract.

	mg. N with added glycine		
	Initial	Final	Change
NH <sub>3</sub> + urea-N	0.08	0.08	0
	0.08	0.11	0.03
NH <sub>2</sub> -N	1.29	0.54	-0.75
	1.38	0.99	-0.39
Amide-N*	0.10	0.10	0
Purine-N	0.38	0.38	0
Total N	1.96	1.90	-0.06
	1.77	1.68	-0.09
Pyruvate*	2.90	1.95	-0.95
	3.84	2.77	-1.07

\* In these exps. kidney slices were used.

addition of NaHSO<sub>3</sub>, used for the estimation of pyruvic acid, presented a difficulty. When the estimation was performed quickly, however, a disappearance of pyruvic acid was observed with added glycine (Table XIV).

It will be recalled that in almost every experiment recorded the O<sub>2</sub> consumption of tissue slices and extracts was slightly inhibited in presence of glycine. As a result of the experiments described with added *Kochsalf*, glycocyamine, pyruvate etc. a theory of condensation of certain factors with glycine has been advanced. Now, most of the substances mentioned are known to increase O<sub>2</sub> consumption when added to tissue in absence of glycine. It seems feasible, therefore, that if a condensation of glycine with such substances occurs, they may be prevented from increasing the O<sub>2</sub> consumption. From Fig. 4 it will be seen

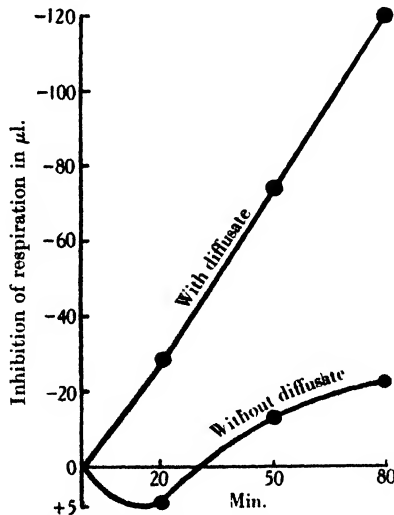


Fig. 4. The effect of tissue diffusate on the inhibition of tissue respiration by glycine.

that the inhibition by glycine is intensified in presence of added tissue diffusate. The latter was prepared by shaking 100 mg. kidney slices in 3 ml. phosphate buffer solution for 2 hr. 1 ml. of this solution was added to another portion of kidney slices suspended in 2 ml. buffer solution.

## DISCUSSION

*The possibility of amino-transfer*

When it was demonstrated in the first part of this work that glycine in absence of any further substrate is not metabolized in tissue extracts, tissue slices or in perfusion experiments with liver and kidney, because no  $\text{NH}_3$  or urea was formed and no  $\text{NH}_2\text{-N}$  disappeared, it must be remembered that similar observations have been made before with other amino-acids, which led to different conclusions. The first observations of Needham [1930], who found an increase in succinic, fumaric and malic acids, but no change in either  $\text{NH}_2\text{-N}$ ,  $\text{NH}_3\text{-N}$ , or amide-N, when glutamic and aspartic acids were incubated with minced muscle, were followed more recently by the experiments of Braunstein & Kritzmann [1937, 1, 2] and on the amino-transfer of amino-acids [Knoop & Oesterlin, 1925]. The former workers observed the formation of glutamic acid from glycine in presence of  $\alpha$ -ketoglutaric acid. No change in the  $\text{NH}_2\text{-N}$  content can be expected under these conditions, i.e. the observations made by these workers are similar to those in the "negative" cases of this experimental work. But it may be argued that had amino-transfer taken place in those cases the result would have been the formation of another amino-acid from a suitable keto compound present, and deamination of this newly formed amino-acid would probably have been the necessary consequence. Thus, kidney tissue containing strongly deaminating enzymes would have given rise to  $\text{NH}_3$ . This was generally not observed in these "negative" cases, although an  $\text{NH}_3$  formation of 8–10% of the  $\text{NH}_2\text{-N}$  added, and a corresponding  $\text{NH}_2\text{-N}$  disappearance of about the same order, occurred in isolated cases. If these small changes in  $\text{NH}_2\text{-N}$  can be considered as significant, the possibility remains that apart from the mechanism suggested by the later experiments, such a deamination either of glycine direct or of an amino-acid formed by the amino-transfer of glycine may take place to a small extent, explaining the findings of other workers who reported  $\text{NH}_3$  formation from glycine of about the same order. These small amounts of  $\text{NH}_3$  have in accordance with these other workers been observed under aerobic conditions only.

The astonishing stability of glycine towards organs, whether extracted, sliced or perfused, gives the impression that a more complicated mechanism than generally attributed to the process of deamination of amino-acids may exist, involving simultaneous contributions by various factors. It seems difficult, therefore, to localize the metabolism of glycine in a single organ without the concept of a contribution by compounds originating in other organs and introduced by the blood stream. This is probably why it was possible to show deamination of glycine administered *per os* or by injection, thus involving the whole metabolic system of the body. Attempts to show *in vitro* effects on glycine incubated with single organs were either frustrated or led to inconsistent results possibly depending on the accidental presence of preformed compounds necessary for the metabolic reaction. The latter could only consistently be observed when metabolites or enzymes of one organ were introduced into the enzymic system of another, thus setting up the necessary system for a metabolic attack on glycine.

*The enzymic nature of the reaction*

The experiments described may give some indication as to whether or not enzymes participate in the reactions observed. For the sake of the following

considerations, it may be permissible to divide the effects of the different biological materials on the disappearance of glycine-N into three groups:

- group — causing a disappearance of 0–10 %,
- group + causing a disappearance of 10–30 %,
- group ++ causing a disappearance of 30–80 %.

The following table summarizes the results of the experiments with different tissues:

1. Kidney slices and kidney extract	—
2. Liver slices and other slices alone	—
3. Kidney + liver slices	—
4. Spleen <i>Kochsaft</i> alone	+
5. Kidney slices + kidney <i>Kochsaft</i>	+
6. Kidney <i>Kochsaft</i> + spleen <i>Kochsaft</i>	+
7. Kidney slices or kidney extract + spleen slices	++
8. Kidney slices + spleen <i>Kochsaft</i>	+

Thus under the experimental conditions described neither kidney nor any other organ alone, nor kidney + liver, is capable without further addition of compounds to cause disappearance of glycine-N (see 1, 2 and 3). Kidney + spleen slices causes a strong effect (see 7). The reaction between glycine and compounds present in spleen *Kochsaft* can take place to a small extent in absence of enzymes (see 4 and 6). The reaction responsible for the disappearance of glycine-N is catalyzed by enzymes present in kidney slices + spleen slices (compare 7 with 4, 5, 6 and 8).

Since in the experiments described the only metabolic change of glycine observed consisted in a disappearance of  $\text{NH}_2\text{-N}$ , a condensation of glycine with a non-enzymic component of the tissue was suggested. Two possible enzymic actions of the tissue may be therefore considered: either a catalytic effect on the mechanism of condensation, or an enzymic action, perhaps involving cozymase, which may lead to the production of tissue components with which the glycine condenses.

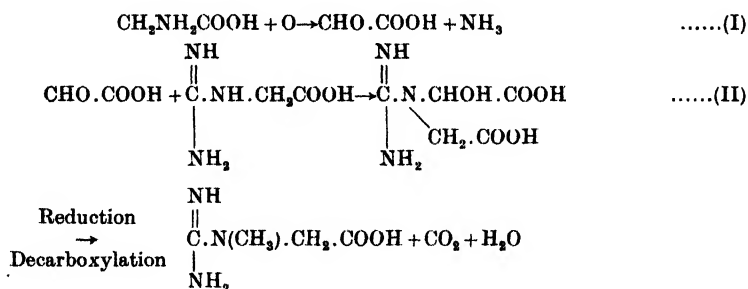
Results of the anaerobic experiments, with cozymase (Table XII) again indicate enzymic action of the tissue, since no effect was observed with kidney slices alone, whereas a definite disappearance of  $\text{NH}_2\text{-N}$  occurred in most experiments with kidney slices and added cozymase. Cozymase may therefore react either with the enzyme which catalyses the condensation, or cozymase may be concerned with the enzymic formation of suitable substrates for the condensation. The first possibility can be excluded by the results of the experiments with pyruvate (Table XIII), as cozymase + pyruvic acid and pyruvic acid alone exert identical effects. For the second question the nature of the non-enzymic components of the condensation has to be taken into consideration.

#### *The nature of the non-enzymic components of the glycine condensation*

(a) *Keto compounds.* These compounds play an essential part in the metabolism of kidney and liver, and condensation of amino-acids with aldehydes and keto bodies is well known to take place purely chemically [Schiff, 1901; Sørensen, 1908]. More recently a similar simple condensation has been described by Herbst [1936] between amino-acids and pyruvic acid. Akasi [1937] reports the isolation of octopine, i.e. a reduced Schiff's base, from muscle of Octopoda: this has recently been synthesized by Knoop & Martius [1938] by condensation of pyruvic acid with arginine.

Pyruvate was shown in fact to cause disappearance of  $\text{NH}_2\text{-N}$ , and here again, the failure to detect a production of  $\text{NH}_3$  and other nitrogenous products of decomposition strongly suggested the formation of a product of condensation of glycine with pyruvate. This suggestion was supported by a disappearance of pyruvic acid simultaneously with the  $\text{NH}_2\text{-N}$  as observed in the cases recorded. The lability of such a condensation product probably prevented the establishment of a clear stoichiometric comparison between the disappearance of  $\text{NH}_2\text{-N}$  and pyruvate. Finally, the fact that the presence of cozymase was required for the reaction with kidney slices alone, but not when pyruvate was added, suggested a link with dehydrogenase systems known to be present in kidney, by means of which keto bodies may be formed. Thus pyruvic acid possibly produced by the lactic-pyruvate dehydrogenase system represents an example of such a reaction. Other keto-compound systems, such as hydroxybutyric-oxaloacetic or more likely the malic-oxaloacetic dehydrogenase, may be taken into consideration, the last being reported to be present in high concentration in kidney [Green, 1936]. Adler *et al.* [1938] consider that the fixation of  $\text{NH}_3$  by keto-acids in animal cells takes place with the help of aspartic dehydrogenase, the activity of which is dependent on cozymase. Although no evidence of a direct link between such dehydrogenase systems and the disappearance of  $\text{NH}_2\text{-N}$  has been produced in this work, it may be pictured that the equilibrium of such a system may be shifted towards the formation of keto compounds in presence of glycine as a fixative for such keto compounds. This problem will be a matter of further investigation.

(b) *Glycocyamine*. The underlying idea of these experiments was the general methylating capability of glycine as shown by a number of workers [Hoppe-Seyler, 1930; Challenger, 1936] and the special methylating action of glycine on glycocyamine to form creatine (*v. supra*). The mechanism of the methylation of glycocyamine by glycine may be pictured to take place as follows:



The condensation of the deaminated glycine with glycocyamine is presented above in a scheme slightly different from that set up by other workers. Glyoxylic acid was considered to be an easier condensing material than glycollic acid which has been postulated by other workers, to act as the methylating agent following the deamination of glycine. The former view is supported by findings of Milhorat & Toscani [1936] who obtained a greater rise in creatine, when glycine was administered to patients with muscular dystrophy than when glycollic acid was used. The scheme postulates as the first step a deamination of glycine. According to Table XI a small  $\text{NH}_3$  production was observed when glycine or glycocyamine was added to heart tissue. Thus this was the only tissue tested in which glycine was shown to produce  $\text{NH}_3$  on incubation. Furthermore, the  $\text{O}_2$  consumption was definitely increased with glycocyamine + glycine whereas

it was decreased with either glycine or glycoeyamine alone. Although the changes mentioned are small, they may support the evidence for the formation of creatine *in vitro*.

#### *General metabolic action of glycine*

It was the object of these experiments to demonstrate the principle of condensation of glycine in place of its simple deamination. Many more possible condensations are conceivable, a number of which are known to take place, such as the formation of hippuric acid by condensation with benzoic acid, the condensation to carbamidoacetic acid [Hill, 1932], by condensation with alloxan, and possibly others. Owing to its instability the product of condensation with keto bodies cannot be regarded as an end-product in the metabolic path. The compound may either undergo tautomerization as suggested by Braunstein & Kritzmann [1937, 1, 2] and Knoop & Martius [1938] followed by splitting with the formation of a new amino-acid, thus completing amino-transfer. Or it may be reduced similarly to octopine and thus be stabilized. In this way glycine may act as fixative for keto compounds producing an equilibrium effect in systems responsible for the formation of such compounds.

The latter phenomenon may lead to a basis of the well-known "specific dynamic effect" of glycine by picturing its role as an acceptor of aldehydes and keto products thus catalysing the reaction within the enzymic systems concerned. By its condensing capacity it may act as a general reagent for the removal of compounds the metabolism of which would otherwise come to a standstill.

#### SUMMARY

1. Glycine, unlike alanine and other amino-acids, is little or not at all deaminated when incubated with slices of kidney, liver, spleen, diaphragm and brain of rat, or with extracts of kidney, testis and other organs. The  $\text{NH}_3$  and urea formation and the disappearance of  $\text{NH}_2\text{-N}$  does not exceed 8% of that corresponding to the amount of glycine added and is not increased by fixatives such as  $\text{NaHSO}_3$  and ornithine. 65–100% of added *dl*-alanine (calculated on the basis of the unnatural isomeride) was deaminated under the same conditions. The deamination of alanine was not increased by the addition of glycine.

No  $\text{NH}_3$  and urea formation was observed during the perfusion of liver and kidney of the cat with glycine. At the end of the experiment all the glycine was recovered as  $\text{NH}_2\text{-N}$ . Glycine was isolated from the perfusion fluid as the  $\beta$ -naphthalenesulpho-derivative.

2. When glycine was incubated with a mixture of kidney and spleen slices or kidney extract + spleen slices, a disappearance of 70–80% of the  $\text{NH}_2\text{-N}$  was observed, without a corresponding formation of  $\text{NH}_3$  or urea. The total N content of the deproteinized fluid was unchanged before and after the experiment. On the other hand a mixture of kidney and liver slices with or without added activators, for example alloxan, caused no such effect. From experiments using kidney slices with added *Kochsaft* of kidney or spleen a condensation of glycine with heat stable compounds under the influence of kidney and spleen enzymes is suggested.

3.  $\text{O}_2$  consumption of tissue slices and extracts is slightly inhibited by glycine. This phenomenon is believed to be caused by its condensation with oxidizable compounds, thus protecting them from oxidation. Under the same



experimental conditions the oxidation of *dl*-alanine corresponds to the theoretical amount required for oxidative deamination of the unnatural isomeride.

4. Glycoeyamine was shown to cause a disappearance of  $\text{NH}_2\text{-N}$  similar to that produced by kidney and spleen *Kochsaft* when added to glycine in presence of mixtures of tissue slices.

5. A small creatine and  $\text{NH}_3$  formation was demonstrated *in vitro* from glycine when incubated with heart tissue in presence of glycoeyamine. A scheme is suggested for the condensation of glycine with glycoeyamine to form creatine.

6. 40–60 % of the  $\text{NH}_2\text{-N}$  disappeared when glycine was incubated with kidney slices or kidney extract in presence of cozymase or pyruvate under anaerobic conditions. The effect was more consistent with pyruvate, which partly disappeared simultaneously, and was not increased with further addition of cozymase. The role of the latter is believed to be concerned with the formation of keto compounds. No change was observed in these experiments in the content of  $\text{NH}_3$ -, amide-, purine- and total N before and after the experiment. Pyruvate, when incubated with glycine in presence of boiled kidney extract under anaerobic conditions, and even in absence of tissue, induced a small disappearance of  $\text{NH}_2\text{-N}$ , but none in the absence of tissue under aerobic conditions.

7. The possible metabolic pathways of glycine are discussed with special reference to amino-transfer. A general tendency of glycine to condense with glycoeyamine, keto-bodies and other compounds is suggested. The influence of cozymase, possibly introduced into the system by *Kochsaft*, may point to a link between dehydrogenase systems concerned with the formation of keto-compounds (pyruvic acid, oxaloacetic acid etc.), and the above-described condensation of glycine with such compounds. Glycine may thus shift the equilibrium in such systems in the direction of increased oxidative production of keto-acids, a possibility that would explain its well-known specific dynamic effect.

I wish to express my thanks to Dr N. G. Myers for his help in the perfusion experiments and to Mr S. Williamson for carrying out part of the  $\text{NH}_2\text{-N}$  estimations. I am grateful to Sir F. G. Hopkins for his kind encouragement during the course of this research.

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# XIII. THE REPRESENTATIVENESS OF EXTRACTED SAMPLES AND THE EFFICIENCY OF EXTRACTION OF PROTEIN FROM THE FRESH LEAVES OF PLANTS; AND SOME PARTIAL ANALYSES OF THE WHOLE PROTEINS OF LEAVES

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ALTHOUGH from the time of Rouelle [1773] a method had existed for the extraction of impure protein samples from the fresh leaves of plants, very little extraction work was done until Osborne & Wakeman [1920] macerated spinach leaves and obtained protein from the juice. Since 1920 most of the work has been done by Chibnall and co-workers, with progressive improvements in technique: Chibnall & Schryver [1921]; Chibnall [1922; 1923; 1924]; Chibnall & Grover [1926]; Miller & Chibnall [1932]; and Chibnall *et al.* [1933].

By repeated maceration of spinach leaves in the presence of water Osborne & Wakeman [1920] extracted all but 16 % of the nitrogen. They centrifuged the green juice to remove cell debris and flocculated two fractions of protein by adding successive portions of alcohol, the fractions, after purification by extraction with solvents, containing 12.9% N and 14.9% N respectively. Subsequently, Osborne *et al.* [1921] fractionated the nitrogenous constituents of lucerne leaves by macerating and extracting with various solvents, and obtained fairly pure specimens of protein by filtering the extracts through paper pulp before flocculating. Of the 56% of the leaf N which was not obtained in aqueous solution very little could be extracted by 93% ethyl alcohol or 0.075 N NaOH solution, but nearly 40% was extracted by 0.075 N NaOH in 60% alcohol. They noticed that the chloroplast material in the aqueous extract was removed by filtration. Considerable protein was flocculated from the filtrates of the aqueous and alkaline water-alcohol extracts.

The attention of Chibnall and co-workers has frequently been directed towards the preparation of protein samples in as high a state of purity as possible. Chibnall [1923] observed that the readily diffusible constituents of the leaf cells could be removed very well by plasmolysing with ether and other anaesthetics and pressing the leaves. Subsequently ether-water and "used" ether-water (i.e. ether-water already used once for the purpose) were substituted for ether, because they permitted better extraction of the protein when the leaves were macerated later in the presence of water. The juices, thus freed in advance from most of the diffusible solutes, were filtered through paper-pulp and the proteins were flocculated, coagulated and purified by extraction with various organic solvents. The yields have varied greatly (from 1 or 2 to 40 % of the leaf protein), but some fairly "pure" preparations have been made. Using ether as plasmolysing agent

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preparations have been obtained from spinach and broad-bean leaves containing 15.95 % N and 1.7 % ash, and 16.05 % N and 4.3 % ash, respectively. These are the highest N contents of any preparations yet obtained from leaves.

With the object of improving the purity of preparations Osborne & Wakeman [1920], Chibnall & Schryver [1921] and Davies [1926] dissolved them in alkali and reflocculated with acid, and were usually successful in increasing the percentages of N. Foreman [1938] obtained small yields of relatively pure preparations by special techniques which did not involve solution in alkali.

There have been two aims in most of this protein extraction work: (1) to obtain large yields, in the belief that high-yield preparations are likely to be more representative of the whole protein in the leaves, and (2) to obtain "pure" preparations because of the difficulties and uncertainties which may attend the analyses of impure preparations. But unfortunately the two aims have usually proved to be mutually exclusive, and it has long been desirable to have some means of checking the representativeness of extracted protein samples. The only accessible means of classifying them and comparing them with the whole leaf-protein of which they represent a part and with the residual leaf-protein (i.e. that not extracted), is through their compositions. An extracted protein preparation may contain some 15 % N whereas, after removal of non-protein N, the whole leaf and the residue may contain only 3 or 4 % N. The methods of analysis employed must therefore be free from interference by very large amounts of impurities, and there are not, as yet, many such methods. Miller [1935] estimated the lysine, arginine and histidine contents of whole and extracted leaf-proteins of cocksfoot grass, and believed that the differences encountered, though fairly large, were of uncertain significance. The object of the present work was to obtain information of the composition of whole leaf-proteins, and to test the representativeness of samples of protein extracted by methods already in use and by any others which suggested themselves.

All materials from which non-protein N has been removed, whether they represent the whole leaf, the extracted preparations or the leaf residues, will be referred to hereafter as "protein preparations" and will be differentiated where necessary by the self-explanatory prefixes "whole", "extracted" and "residual" and the corresponding abbreviations W, E and R.

## EXPERIMENTS AND DISCUSSION

### *The preparations (general)*

Details concerning the fresh leaf materials and the extraction of samples of protein are contained in Tables I and II. The contents of Table II are explained later in the text and in accompanying remarks. Table I shows in the order of the columns: the label of the protein preparation (exp. number, followed by abbreviation showing whether "whole", "extracted" or "residual" and sometimes other distinguishing signs): the leaf-material source and the date: the percentage dry weight of the leaf material: the percentage of total N (*t*), coagulable N (*c*) and protein N (*p*) in the dry leaf: abbreviations indicating the method of extracting the sample of protein (if an extracted preparation): the percentage of the leaf N (*t*, *c* and *p*) appearing in an extracted juice or leaf residue: the percentage of N in the dry protein preparation; and finally the percentage of ash in it after ignition in air at about 600°. Sometimes a few % more or less total N could be found in the juice plus residue than was calculated to have been present in the fresh leaf material used. As there was never any definite evidence of real loss of N

Table I

Prep.	Source and date	% dry wt.	% N in dry leaf (t, c, p)	Method of extraction	% extracted and residual N (t, c, p)	% N in prep.	% ash prep.
1W	<i>Dactylis glomerata</i> (cocksfoot grass)	29. ix. 36	6.81t, — 4.50p	—	—	7.53	3.2
1E	"	"	"	w, c, w, p, , f.	—	14.1	1.1
2W	"	7. vi. 37	4.05t, — 3.36p	—	—	5.61	5.6
2E(a)	"	"	"	w, c, w, p, , f.	—	13.05	1.2
2E(b)	"	"	"	d, fs, w, p, , f.	71.5t, — 61.1p	12.6	1.4
3W	<i>Lolium perenne</i> (perennial rye-grass)	9. vi. 37	4.19t, — 3.38p	—	—	4.93	23.2
3E(a)	"	"	"	w, c, w, p, , f.	—	13.55	1.4
3E(b)	"	"	"	d, fs, w, p, , f.	—	12.35	6.4
4W	"	29. xi. 37	4.20t, 3.14c, 3.00p	—	—	5.12	13.3
4E'	"	"	"	d, c, pH 7, p, , c.	45.1t, 29.4c, 27.8p	13.1	7.3
4E''	"	"	"	, , pH 10, p, , c.	4.1t, 3.2c, 3.1p	8.15	25.8
4R	"	"	"	—	50.8t, 63.7c, 62.7p	3.45	19.9
5W	<i>Beta cicla</i> var. (beet spinach)	3. xii. 37	4.17t, 3.17c, 3.02p	—	—	6.14	9.7
5E'	"	"	"	d, c, pH 7, p, , c.	46.9t, 30.7c, 29.4p	13.3	6.3
5E''	"	"	"	, , pH 10, p, , c.	4.6t, 3.1c, 2.9p	12.1	7.6
5R	"	"	"	—	48.5t, 60.4c, 58.9p	4.06	12.8
6W	<i>Festuca ovina</i> (sheep's fescue grass) [See Table II]	19. v. 38	3.14t, 2.52c, 2.41p	—	—	—	—
6							
7W	<i>Lolium perenne</i> (perennial rye-grass)	8. vi. 38	4.43t, 3.60c, 3.40p	—	—	5.77	4.7
7E	"	"	"	d, c, pH 9, sc, , .	56.2t, — 46.5p	12.5	3.7
7R	"	"	"	—	43.8t, — 48.5p	3.54	5.6
7E1	"	"	"	d, c, pH 9, sc, ae, c.	50.7t, — 46.1p	13.2	3.0
7E1	"	"	"	—	5.5t, — 4.6p	8.5	—
7E2	"	"	"	d, c, pH 9, sc, ae, f.	36.8t, — 30.2p	14.65	0.6
8W	<i>Dactylis glomerata</i> (cocksfoot grass)	4. x. 38	7.34t, 6.16c, 5.92p	—	—	8.91	3.9
8E1	"	"	"	d, f, pH 9, sc, , .	92.1t, 86.7c, 85.9p	13.25	3.1
8E2	"	"	"	d, f, pH 9, sc, ae, .	92.1t, 94.1c, 94.1p	13.4	3.8
8R	"	"	"	—	7.9t, 8.8c, 8.4p	1.82	7.8
8E3	"	"	"	d, f, pH 9, sc, , f.	69.1t, 60.3c, 59.7p	14.2	0.6
8E4	"	"	"	d, f, pH 9, sc, ae, c.	87.5t, 89.0c, 88.8p	14.55	2.2
8RE4	"	"	"	—	4.6t, 5.2c, 4.7p	6.16	—
8E5	"	"	"	d, f, pH 9, sc, ae, .	65.6t, 62.5c, 60.7p	15.0	0.6

Table II

8 g. portions of fresh leaves of *Festuca ovina* (22.7% dry wt.; 3.14% total N, 2.52% coagulable N and 2.41% protein N in the dry leaves) were macerated in the end-runner mill for 5 min. with 50 ml. of (i) water; (ii) pH 7.2 sodium phosphate buffer containing 1.5 g. P per litre; (iii) and (iv) pH 9.2 sodium borate buffer containing 1.1 g. B per litre; and the mash was centrifuged for 2 min. at 100 × gravity. From moisture content of leaves and moisture loss during maceration (estimated by tests with standard acid), the final volume of liquid was calculated to be 55.0 ml. Each 5 ml. therefore should have contained 5.17 mg. total N, 4.15 mg. coagulable N and 3.98 mg. protein N if all the N had been completely and homogeneously dispersed into the liquid. Compare with data in column (2).

In (ii), (iii) and (iv), the 5 ml. aliquots of juice (left at pH 6 if water had been used, otherwise adjusted with alkali to buffer pH value) were centrifuged for about 30 min. at 25,000 × gravity to separate the granule from the non-granule fractions, judged by transparency of, and absence of "greenness" in the liquid layer. See columns (3) and (4). The granules, when later extracted with a protein solvent, were stirred with about 5 ml. of the solvent and the emulsion was left for 10 min. before recentrifuging. See columns (5)–(16). Data in column (17) were obtained from mixed extracts of the granules as indicated. The solvents were variously: 5 ml. of the aqueous solvents, water, diluted pH 7.2 buffer, diluted pH 9.2 buffer, and 0.05 N NaOH; 4.5 ml. of aqueous solvent plus 0.5 ml. of ether; 2.5 ml. of aqueous solvent plus 2.5 ml. of alcohol; and 2.5 ml. of aqueous solvent plus 2.0 ml. of alcohol and 0.5 ml. of ether.

In (iv) the juice itself was treated with a mixture of 0.8 vol. of alcohol and 0.2 vol. of ether before centrifuging. The figures in parentheses above certain values indicate the percentages of N in the dried precipitates concerned. The granule N: non-granule N ratio in (i) is believed to have been too high from sedimentation of a little non-granule protein (denatured?) as a pale top layer.

a non-granule protein (denatured) as a pure top layer.

Form of N (1)	Juice Mg. (2)	Non-gran.		Action of solvents upon granules												Insol. N mg. (16)	Sum of N mg. 5, 7 and 9 (17)										
		N mg. (3)	Gran. N mg. (4)	Water				Water ether				pH 7.2 buffer						Insol. N mg. (10)	Sol. N mg. (11)	Insol. N mg. (12)	Sol. N mg. (13)	Insol. N mg. (14)	Sol. N mg. (15)				
				Sol. N mg. (5)	Insol. N mg. (6)	Sol. N mg. (7)	Insol. N mg. (8)	Sol. N mg. (9)	Insol. N mg. (10)	Sol. N mg. (11)	Insol. N mg. (12)	Sol. N mg. (13)	Insol. N mg. (14)	Sol. N mg. (15)													
Total	4.69	2.96	1.73	Water				Water ether				pH 7.2 buffer				1.55	—	—	—	—	—	—	0.51	—			
Coagulable	3.50	—	—	0.14	1.59	0.21	1.52	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Protein	3.35	1.74	1.61	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.18
(ii)		pH 7.2 buffer				pH 7.2 buffer				pH 7.2 buffer				pH 7.2 buffer													
Total	4.93	3.24	1.69	0.21	1.48	0.44	1.25	0.57	1.12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.22
Coagulable	3.80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	(12.0)
Protein	3.63	(11.7)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.93
(iii)		pH 9.2 buffer				pH 9.2 buffer				pH 9.2 buffer				pH 9.2 buffer				pH 9.2 buffer									
Total	5.01	3.27	(7.7)	0.34	1.40	0.67	1.07	0.97	0.77	1.20	0.54	0.53	1.21	1.35	0.39	1.96	—	—	—	—	—	—	—	—	—	—	—
Coagulable	3.86	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Protein	3.68	(12.2)	(11.3)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	(13.0)
(iv)		pH 9.2 buffer add alcohol ether				pH 9.2 buffer				pH 9.2 buffer				pH 9.2 buffer													
Total	5.01	4.41	(4.6)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Coagulable	—	—	0.58	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Protein	3.89	(14.5)	0.47	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

(as  $\text{NH}_3$ , for example), the discrepancies were attributed to sampling errors. Values shown in the table have been adjusted as for perfect recovery of total N.

Of the abbreviations concerning the method of extracting protein from the leaves, *d* signifies direct maceration with the protein solvent, or *w*, maceration after removal of readily diffusible leaf-cell constituents by plasmolysis with "used" ether-water and pressure in the Chibnall *et al.* [1933] process. Following these abbreviations, *c* means that the material was coarsely macerated in a mincing-machine, *f*, that it was finely macerated in an end-runner mill, or *fs*, that it was finely macerated with 20% by weight of sand in the runner mill. Then *w* signifies that water, or "*pH* 7 (or 9 or 10)" that a buffer solution of the indicated *pH* value, was the protein solvent with which the leaves were macerated. Following these, *p* signifies that the mash was pressed in a cloth, or *sc*, that it was sieved and the liquid centrifuged for 2 min. in a field of  $100\times$  gravity, to separate the juice from most of the cell debris. Then *ae* indicates that a cold mixture of 0.8 vol. alcohol and 0.2 vol. ether was stirred into the juice with cooling and the mixture allowed to stand for 15–30 min. Finally, *f* means that the juice was filtered through a pad of paper-pulp, or *c*, that it was centrifuged for 30 min. in a field of  $500\times$  gravity, before the protein was flocculated. Blanks in the sequence of abbreviations indicate omitted steps. The extractions were done at room temperature (10–25°).

In general, a higher percentage dry weight of leaf in any one species reflects greater age of leaf; and, ages being similar, a higher percentage of N in the dry leaf reflects better manuring. Inasmuch as it is believed that the leaf proteins of the species would all contain some 17.0% N if they could be obtained pure, the actual values for the preparations afford some measure of purity, and, in conjunction with the percentages of ash, some measure of organic impurity. It is believed that nitrogenous impurities were absent from the protein preparations.

#### *Methods of estimation*

Nitrogen estimations were made by the Kjeldahl method. The terms "coagulable nitrogen" and "protein nitrogen" have been explained in earlier articles [Lugg, 1938, 2, 3] and apply in the present article to the original leaf materials, to the extracted juices containing protein and other nitrogenous substances, and to the leaf residues after extracting juices. Briefly, "protein N" means N not extracted by successive applications of boiling alcohol, dilute citric acid, alcohol and ether, to a material containing "coagulable N"; and (usually) "coagulable N" means N left in insoluble material after heating at about 90° in dilute acetic acid at about *pH* 4.5. In some instances, to be discussed, coagulation was performed by heating to about 70° in water-alcohol mixtures (which sometimes contained ether until it had been boiled off) at a *pH* corresponding with that of a mixture of aqueous acetate *pH* 4.5 buffer with 0.8 vol. alcohol, and therefore above 4.5 but on a different *pH* scale (see, e.g. Richardson [1934]). By coagulating in presence of 0.8 vol. alcohol it was normally found that the coagulable N and protein N were increased in the case of a juice, but in the case of a dried leaf material they were almost unaffected as also were the percentages of N and ash in the resulting whole protein preparation.

Amide, tyrosine and tryptophan, and cystine plus cysteine and methionine estimations were made with the protein preparations by the methods described in the articles [Lugg, 1938, 1, 2, 3], wherein it has been shown that the estimations of amide, tyrosine, tryptophan, cystine plus cysteine (by differential oxidation) and methionine (by differential oxidation) are not subject to fresh systematic errors when some carbohydrates are added to a protein. It was

assumed that they would be satisfactory in the presence of much cellulose and other cell-wall constituents; and, in fact, satisfactory tests of the procedures for estimating tyrosine and tryptophan were made with much cellulose present. It was found too that amide values obtained with the whole and residual protein preparations by varying the hydrolysis procedure differed from those obtained by the standard procedure in much the same way as did values with extracted protein preparations [Lugg, 1938, 3]. Estimations with whole and residual protein preparations were sometimes made on a larger scale than was customarily adopted with extracted protein preparations.

The random errors of estimation with whole and residual protein preparations are believed to have been not more than about 1.5 times as great as they were in the case of extracted protein preparations, and these last are believed not to have exceeded the figures indicated in the following general description of Table III,

Table III

Prep.	% N	% amide N	% tyr.-N.	% try.-N	% cyst.-N (d.o.)	% meth.-N (d.o.)	% cyst. + meth.-N (d.o.)	SO <sub>4</sub> -S × 100/N (d.o.)	% cyst.-N (HI t)	% meth.-N (HI t)
1W	7.53	5.10	2.44	1.90	1.38	1.46	2.84	1.07	—	—
1E	14.1	5.26	2.34	1.82	1.68	1.49	3.17	0.40	1.50	1.30
2W	5.61	5.04	2.53	1.78	1.41	1.61	3.02	0.36	—	—
2E(a)	13.05	4.95	2.32	1.80	1.55	1.42	2.97	0.78	1.35	1.22
2E(b)	12.6	5.02	2.39	1.88	1.44	1.52	2.96	0.65	—	—
3W	4.93	5.26	2.48	1.82	1.41	1.48	2.89	0.39	—	—
3E(a)	13.55	4.73	2.34	1.73	1.69	1.58	3.27	0.66	1.54	1.50
3E(b)	12.35	5.15	2.44	2.09	1.57	1.53	3.10	0.25	—	—
4W	5.12	5.20	2.41	1.78	1.47	1.59	3.06	0.76	—	—
4E'	13.1	4.96	2.43	2.05	1.63	1.52	3.15	1.12	1.20	1.07
4E''	8.15	5.64	2.28	1.62	1.48	1.49	2.97	1.30	—	—
4R	3.45	5.15	2.44	1.54	1.51	1.58	3.09	1.02	—	—
5W	6.14	5.28	2.63	1.50	1.45	1.43	2.88	0.63	—	—
5E'	13.3	5.18	2.57	2.01	1.47	1.44	2.91	0.91	1.24	1.17
5E''	12.1	5.18	2.68	1.71	1.59	1.39	2.98	0.73	—	—
5R	4.06	4.97	2.79	1.35	1.53	1.42	2.95	0.94	—	—
7W	5.77	5.21	2.45	1.92	1.42	1.50	2.92	0.62	—	—
7E	12.5	5.27	2.42	1.97	1.40	1.60	3.00	0.43	1.21	1.41
7R	3.54	5.30	2.51	1.88	1.48	1.45	2.93	0.74	—	—
7E1	13.3	5.15	2.35	1.92	1.46	1.47	2.93	0.34	1.17	1.36
7E2	14.65	5.17	2.39	1.96	1.37	1.46	2.83	0.34	1.25	1.40

which contains the results of analyses. In the order of the columns are shown: the characteristic label of the preparation: the percentage of N in it (error 0.5 %, affecting all the other errors): the amide content (error 1.5 %): tyrosine content (error 2 %): tryptophan content (error 3 %): cystine plus cysteine content, by differential oxidation (error 4 %): methionine content, by differential oxidation (error 5 %): the sum of the last two values (error 2 %): sulphate-S as a percentage of the weight of the N in the preparation (error from 3 % for the higher to 20 % for the lower values): cystine plus cysteine content, by titration of HI digests (error 3 %): and finally the methionine content, by titration of HI digests (error 4 %). The values have been corrected for such losses as the pure, free amino-acids undergo in the analytical procedures involved. To facilitate comparison of the compositions by way of proving the non-identity or assessing the probability of identity of the protein moieties of preparations (whole, extracted and residual), the results have been reported on a nitrogen basis, namely, as percentages of the N in the preparations appearing as the particular chemical species (amide or amino-acid) estimated. Although they are believed not to have



contained nitrogenous impurity, the preparations may have contained N in groups (such as nucleic acid) conjugated with some of the protein. Values in the last three columns cannot be used in comparing compositions. Those in the last two are probably low, though the precise significance of the disparities between them and the values obtained by the highly unspecific differential oxidation procedure is uncertain, whilst those in the previous column (sulphate-S) are to be regarded as measures of impurity.

(1) *Extraction test with Dactylis glomerata*

A check was made of the representativeness of protein extracted from a batch of *Dactylis glomerata* leaves by the method of Chibnall *et al.* [1933]. Details concerning the fresh leaf material (which was young and taken from a well-manured plot), and the whole and extracted protein preparations, are contained in Table I under the designations 1W and 1E. The results of partial analyses of the preparations are shown in Table III under the same designations. The S distributions were significantly different, the cystine plus cysteine content of 1E being considerably greater than that of 1W.

(2, 3) *Extraction tests with Dactylis glomerata and Lolium perenne*

The object of making the preparations 2W, 2E (a) and 2E (b) from leaves of *Dactylis glomerata*, and 3W, 3E (a) and 3E (b) from leaves of *Lolium perenne*, was to test again the representativeness of protein extracted by the method used in experiment 1 (giving now 2E (a) and 3E (a)) and further, to test the efficacy of a direct fine-maceration method which yielded 2E (b) and 3E (b). The surfaces of the *Lolium perenne* leaves were contaminated with soil. Details concerning the leaf materials and the extraction work are recorded in Table I, and analyses of the preparations in Table III. 2E (a) was of distinctly lower tyrosine content than 2W. Of 3E (a) and 3E (b), the former contained larger amounts of the sulphur-containing amino-acids and less amide than 3W, whilst the latter differed from 3W most noticeably in possessing a higher tryptophan content. Clearly, no significant improvement in the representativeness of extracted protein was achieved by macerating the leaves very finely; and, despite extensive extraction, the leaf residues having been macerated repeatedly with more water, considerable amounts of the leaf proteins were missing from the extracted preparations.

(4, 5) *Extraction tests with Lolium perenne and Beta cicla*

The unsatisfactory recoveries of protein in the fine-maceration tests in Exps. 2 and 3 suggested that much protein was being retained by the filter pads, possibly in association with the plastids and nuclei. In Exps. 4 and 5 (Table I) with leaves of *Lolium perenne* and *Beta cicla* respectively, it was accordingly decided to centrifuge, instead of filter, the juices which had been pressed through a cloth. The juices were centrifuged for 30 min. in a field of 500 × gravity, and although considerable chloroplast and other material was thus removed with cell-wall debris, the juices were still opalescent and strongly green in colour. A direct, coarse-maceration procedure was used in both experiments, and the initial extracting solvent was a pH 7 phosphate buffer containing 1.5 g. P per litre, providing preparations 4E' and 5E' respectively. The residues were then extracted with a pH 10 borate buffer containing 1.1 g. B per litre, but without further maceration, and yielded 4E'' and 5E'' respectively. It was hoped that

such nuclei, chloroplasts etc. as had been liberated from the cells but remained entangled with the leaf residues after the first extraction, would yield at least some of their protein to a pH 10 buffer. The high ash contents of the whole (4W and 5W), the extracted (4E' and 4E'', and 5E' and 5E'') and the residual (4R and 5R) protein preparations, are attributable to the fact that the leaf surfaces were contaminated with loamy soil.

The partial analyses of the preparations are recorded in Table III. They show that the proteins extracted at pH 10 differed considerably from those extracted at pH 7, and that the total extracted proteins were far from being representative of the whole proteins of the leaves. In neither case was the extracted plus residual protein N as great as the whole protein N. The losses were at first attributed to proteolytic activity in the extracts, but it was found later that the amounts of protein left in aqueous solution at pH 4.5, even after heating to 90°, are usually by no means negligible.

The one satisfactory aspect of these two experiments, 4 and 5, was that after making rough corrections for protein losses, all of any particular chemical species estimated in the whole proteins could be accounted for in the extracted and residual proteins to within the random errors of analysis.

#### (6) *Extraction tests with Festuca ovina*

A closer examination of the natures and reactions of the morphological units of the leaf cells was demanded by the findings of this earlier work, and a series of experiments was designed accordingly.

The proteins of plants are almost entirely intracellular, and in living, somatic cells appear to occur mostly in the protoplasm: only in ripening and drying seeds has much protein been found in the vacuoles. The protoplasmic protein is distributed between the nuclei, the ground mass of the cytoplasm and the more solid particles such as the plastids and mitochondria (or chondriosomes). Crystalline and amorphous protein particles may be present free in the vacuoles and cytoplasm, and embedded in the nuclei and plastids. The possibility cannot be ignored that the protein or proteins coming into each of these categories may differ from those in any other, and there may be differences as between one cell and another. Menke [1938], extending the work of Noack [1927], has shown that nuclei and chloroplasts may be separated from the rest of the protoplasm fairly readily by centrifuging and by other means; and Fauré-Fremiet [1913] found that mitochondria could be removed by centrifuging. The protoplasm is thus to be regarded as consisting of fluid and more solid fractions but no simple embracing terms are in use in reference to the entire, mixed cell contents. For simplification of subsequent descriptions and discussions, the terms "non-granule" will be applied to the mixed liquid fractions of vacuoles and protoplasm, and "granule" to the nuclei, plastids, mitochondria, membranes etc.

As a solvent for the protein contained in chloroplast material which had been extracted with alcohol and ether, Menke [1938] employed a reagent used by Osborne *et al.* [1921] and later by Davies [1926], namely, 0.075 *N* NaOH in 60% alcohol-40% water solution. It is probable that the nitrogen extracted from leaf residues by the earlier workers with this reagent was largely of chloroplast origin. Menke [1938] sometimes used dilute aqueous NaOH solutions as solvents for leaf proteins, and they have been used also by Kiesel *et al.* [1934] and by earlier workers. These strongly alkaline reagents, however, could scarcely be expected not to degrade any protein dissolved in them (see e.g. Blish & Sandstedt [1929-1930] and Neglia *et al.* [1938]).

In the earlier experiments with *Festuca ovina*, it was found that by macerating the leaves very finely in a runner-mill with water, or better with a pH 7 or 9 buffer solution, and then centrifuging the mash for a short time in low-intensity fields, the forms of leaf N (total, coagulable and protein) were distributed through the fluid in macro-uniformity and almost in the proportions occurring in the leaves, and very little unextracted N remained in the particles of cell debris which, together with some of the fluid, formed the lower layer of loose sludge. It was otherwise if any attempt were made to separate the debris by pressing the mash in a cloth, for the debris itself acted as a filter to retain preferentially some of the coagulable and protein N, an effect which could be correlated with a visible retention of chloroplast material. From the granules separated by centrifuging in high-intensity fields, it was found possible to extract protein by emulsifying with quite mildly alkaline buffers and adding ether, alcohol and alcohol-ether mixtures; but if ether were ever present in such quantity as to permit a separation of liquid layers the chloroplast material was largely carried up with the ether and the protein in it rendered rather insoluble in the buffer, presumably by denaturation. Furthermore, if very large proportions of alcohol were added the buffer solutions failed to be satisfactory protein solvents.

Typical later experiments with *Festuca ovina* leaves are described in Table II with its accompanying explanation. Of particular interest is the fact that the alcohol-ether-pH 9.2 buffer mixture proved to be almost as effective in dissolving protein from the granules as was the alcohol-NaOH solution mixture. It disintegrated the granules (or at least the chloroplasts), the residues of which retained almost all their chlorophyll and apparently most of their lipin and centrifuged down much more cohesively than undisintegrated granules do. Microscopically, the disintegration was correlated with a tendency of the chloroplasts to lose their shape, to stick together and to smear on glass. From suspension in the buffers themselves, the denser nuclei centrifuged down as a small cream-coloured layer beneath the centrifuged chloroplasts, very much as they did from suspension in water [cf. Menke, 1938], but when the lipid solvents were included with the buffers and the disintegration was pronounced, the cream-coloured layer could not be distinguished. The nuclei and mitochondria may have suffered a fate similar to that of the chloroplasts or they may have been carried down almost intact with the disintegrated chloroplasts. Some protein was obstinately retained by the disintegrated granule residues and it is not known to what morphological units it belonged, though it would probably have included any denatured protein, such as might have been present in dead cells.

Inasmuch as some N could be extracted from the granules without the aid of lipid solvents, it would seem that they might yield much of their protein to water or to plain mildly alkaline buffer solutions if they could be disrupted mechanically to a sufficient degree. It must have occurred to some extent during the grinding operations which yielded the juices examined.

As to the nature of leaf-cell nucleoproteins, the work of Belozersky & Chigirev [1936] and Belozersky & Dubrovskaya [1936] suggests that the protein parts proper are unlikely to be of strongly basic character like the protamines and histones.

Exp. 6(iv) showed that the granule disintegration could be carried out in the presence of the rest of the protoplasmic and vacuole material. From a comparison of the N contents of the relevant protein micro-preparations, it seemed too that the lipid solvents could change the state of hydration or aggregation of certain substances in the juice (mucilages?) in such a way that they could be removed in part with the disintegrated granules by centrifuging in high-intensity fields, thus

reducing the extent to which they were able later to contaminate the flocculated protein. It will be noted that the proteins were more completely flocculated and coagulated from their solutions if alcohol was present, some of the extra "protein" obtained possibly being of proteose or similar nature.

(7) *Large-scale coarse-maceration extraction of Lolium perenne*

The presence of a mildly alkaline buffer during maceration of the leaves is favoured by the considerations that a fairly satisfactory solvent for solid (deposit) protein is thereby provided, that the other units comprising the granule fraction of the cell contents appear to suspend or emulsify in it better than they do in more neutral media, and that leaves which have so strongly acid a cell-sap that they fail to yield even their non-granule protein in solution when macerated with water might be expected to behave towards the buffer much as the *Festuca ovina* leaves behaved (cf. Chibnall & Grover [1926], who used dilute NaOH solutions to extract such leaves). In regard to the alkalinity of the buffer, the reversible-dissociation ranges of the proteins concerned should not be exceeded. It might be safe to use buffers at pH 10 or even pH 11. At all events, such evidence as does exist suggests that irreversible dissociation does not normally occur with proteins in the neighbourhood of pH 9, in which region the degree of reversible dissociation is frequently negligible (see e.g. Svedberg & Sjögren [1930, 1, 2]; Sjögren & Svedberg [1930, 1, 2]; and a recent review by Svedberg [1937]). Subsequent addition of lipid solvents would decrease the ionization constants of buffer acids (thereby normally increasing the pH, but on a different pH scale) and the dissociation and ionization constants of the proteins would probably be modified too, but the resultant effects in regard to protein dissociation are not known.

The above considerations apply whether the leaf material is macerated finely or coarsely. By repeated fine maceration and avoidance of any loss of the granule fraction it would seem from experiment 6 that almost all the protein could be extracted and obtained as a preparation which might not be very pure but must be almost perfectly representative, and that at some sacrifice of representativeness purer preparations could be obtained with the aid of lipid solvents. The large-scale extraction of leaf-protein samples, however, can be effected more easily by coarse than by fine maceration procedure. Many of the leaf cells are not ruptured by coarse maceration and those that are ruptured may not yield their contents to the juice in proper proportion if the maceration procedure itself involves a partial filtration by extrusion of the contents through split cell-walls. In principle, therefore, coarse maceration should amount to random clean cutting or tearing, without compression, and the requirements are fulfilled to a degree by a mincing-machine though not perfectly. *Lolium perenne* was one grass from which it had been lamentably easy to extract unrepresentative protein and it was accordingly chosen to provide fairly rigorous tests of procedures based upon the findings of Exp. 6 and adapted to large-scale extraction with the aid of the mincing-machine.

The leaf material was of medium age and had been grown in a rather shady environment. By finely macerating a small portion with a pH 9 buffer and centrifuging the juice as in Exp. 6, 32.8% of the leaf total N was found to be associated with the granule fraction containing all the visible chlorophyll. The main portion was passed through the mincing machine with three times its weight of a pH 9.2 sodium borate buffer containing 1.1 g. B per litre, the juice was separated from cell debris by violent shaking and stirring on a wire sieve (holes 1 mm. sq.) followed by centrifuging for 2 min. in a field of 100 × gravity,

and the residues were washed by stirring up with more buffer. The mixed juices were adjusted to pH 9.2 and divided up to give preparations 7E, 7E1, 7E2 and 7RE1 in Table I, 7RE1 representing disintegrated granule residue separated by the centrifuge before flocculating 7E1. The data have been calculated back as if all the juice had been used for each preparation. It should be noted that 7R, 7E1 and 7RE1 together contained virtually all the protein N calculated to have been present in the fresh leaves. 7E not having been flocculated and coagulated in presence of alcohol, some protein N (calculable from 7E1 and 7RE1 as about 10%) remained in solution in the extract, and gives the impression that much more non-coagulable N in proportion to coagulable N than was actually the case was extracted from the leaves. So too in the earlier extractions (Exps. 1, 2, 3, 4, and 5) must the proportions have been rather less unfavourable than they seemed. In the case of 7E2 there was considerable retention of protein N (and coagulable N) by the filter-pad but not much retention of non-coagulable N. It is one of the purest preparations which have been made from leaves of this grass. Whilst 7E might have been expected to contain too little of the non-granule protein, the reverse might have been expected of 7E1.

The whole, extracted and residual protein preparations (except 7RE1) were analysed, and the results are reported in Table III under 7W, 7E, 7E1, 7E2 and 7R. They showed, to within the limits of the random errors of analyses, no evidence of unrepresentative extraction of protein in any of the three preparations obtained: a most gratifying result in view of the hypothetical difficulties discussed. Apparently the large amount of protein retained by the filter-pad in the preparation of 7E2 was itself moderately representative of the whole protein.

#### (8) *Small-scale fine-maceration extraction of Dactylis glomerata*

Experiments were made with *Dactylis glomerata* with the object of generalizing further, if possible, some of the findings made with *Festuca ovina* (6) and *Lolium perenne* (7), and at the same time of obtaining high-efficiency extraction of the protein on a larger scale than in Exp. 6. 100 g. of fresh leaves of young-medium age, estimated by the method used in Exps. 6 and 7 to contain 33.1% of the total N in the granule fraction, were macerated in several portions in the runner-mill with a pH 9.2 borate buffer and the juice was separated from the residues with the aid of the sieve and the centrifuge as in Exp. 7, the residues being treated similarly twice in succession. The mixed juices, occupying a volume of nearly 1 litre, were adjusted to pH 9.2 and divided up for the preparation of 8E1, 8E2, 8E3, 8E4 (with 8RE4) and 8E5 as shown in Table I, the data being calculated back as if the entire amount of juice had been used for each preparation. 8RE4 represents the disintegrated granule material separated by the centrifuge before flocculating 8E4.

In general the results confirmed those found with the other plant species in Exps. 6 and 7. Again it was seen that the flocculation and coagulation of the protein in the juice were assisted by the presence of alcohol. In fact the recoveries of the whole (8W) coagulable N and protein N between the extracted and residual material, 8E2 and 8R or 8E4, 8RE4 and 8R, were a little in excess of 100%, and suggested that in drying the leaf material at 80° some 2% of the protein N had been converted into non-coagulable N. Just as in Exp. 6, centrifuging in a field of 500 × gravity (8E4) was less effective in preferentially removing contaminating substances from the juice containing alcohol and ether than filtration (8E5) was, but a field of 25,000 × gravity might have been as effective had it been possible to employ the high-speed centrifuge with such quantities of juice. Much protein was retained by the filter-pads whether alcohol and ether were

present (8E5) or not (8E3), but the former preparation, which appears to be the purest yet made from leaves of this grass, was considerably purer than the latter and was, presumably, more nearly representative of the whole leaf-protein. 8E2 must have been highly representative, whilst 8E1 may have been slightly deficient in the non-granule fraction. Of the total N in the residue 8R, rather too small a fraction in comparison with that of the original leaf material, was non-coagulable, suggesting that the residue contained insufficient non-granule protein to be representative of the whole. On the other hand it contained only 8.4% of the original protein N.

It is presumed that preparations similar to 8E4 and 8E5 could have been obtained by macerating the leaves in the presence of the lipid solvents, but the general effects of having lipid solvents present at this stage were not investigated.

#### *Further considerations*

Local acidity, causing differential flocculation of some of the non-granule proteins, is the only obvious hypothesis to be advanced in explanation of the rather pronounced differences in composition between 3E(a) and 3E(b). In passing it should be mentioned too that at pH values below 6 the non-granule proteins are usually very prone to surface denaturation. In the absence of salt their solutions in dilute HCl on the acid side of the isoelectric regions may frequently be boiled without appreciable coagulation.

It is of interest to compare the partial compositions of the whole proteins of the three batches of *Lolium perenne* leaves (3W, 4W and 7W), and those of two batches of *Dactylis glomerata* leaves (1W and 2W). The differences are small and of the most doubtful significance. In the first place, age of the leaves and climatic and manurial conditions and locality of growth appear to have been without appreciable effect upon composition of the whole leaf-proteins. In the second place there is no evidence that the whole leaf-proteins of the two species from the order *Gramineae* differed in composition. The whole leaf-protein of the single batch of *Beta cicla* from the order *Chenopodiaceae*, on the other hand, differed from these in having a lower tryptophan and a higher tyrosine content.

Even when appropriate precautions were not taken during extraction and the extracted proteins were demonstrably unrepresentative of the whole leaf-proteins, the differences in composition were not very great. Previously reported partial compositions of extracted protein preparations [Lugg, 1938, 2, 3] are therefore unlikely to have been seriously different from those of the whole proteins of the leaves from which they were obtained.

#### SUMMARY

Samples of protein extracted from plant leaves in various ways have been examined for representativeness. Points favouring the use of mildly alkaline buffers, when macerating leaves with the object of dispersing the protein-containing cell-units into the juice, and the difficulties of avoiding preferential loss of the proteins belonging to the "granular" units (nuclei, plastids, mitochondria etc.) when subsequently removing cell-wall material and other impurities, have been examined and discussed. It has been shown that the addition of lipid solvents, such as alcohol and ether, to the mildly alkaline juice permits most of the protein of the granule fraction to pass into solution: and the use of such solvents in the preparation of purer samples of protein without serious sacrifice of representativeness, has been demonstrated. Incidentally, the proteins

are much more completely flocculated by acid in their isoelectric regions and coagulated by heat, if alcohol is present in the solutions containing them.

The amide, tyrosine and tryptophan contents and the S distributions (cystine plus cysteine and methionine contents) of the whole proteins of leaves, provided no evidence of variation in composition with the age of the leaves or the manurial and climatic conditions or locality of growth; but composition may vary with plant species.

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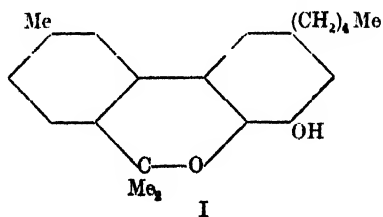
# XIV. THE ACTIVE PRINCIPLES OF *CANNABIS* *INDICA* RESIN. I

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THE resinous exudate of the female flowers of *Cannabis indica* (*C. sativa*) forms the essential constituent of the drug variously known as hashish, bhang, charas, ganja and marihuana according to the country of origin and mode of preparation. Extracts of the flowers have been used in European medicine to some extent but were found to be so variable in activity that *C. indica* has been removed from the *British Pharmacopoeia*. The plant is, however, well known through its use as a drug in oriental countries and recently, under the name of "marihuana", its use has assumed dangerous proportions in America. *Cannabis* resin has been the subject of many investigations in the past but much of the earlier work is contradictory. The active principle is contained in a high-boiling resin and is not an alkaloid [Smith, 1857]. The active fraction isolated from the crude resin by Wood Spivey & Easterfield [1896] and given by them the name cannabinal was later found by the same workers [1899] to be a mixture, and the name cannabinal was transferred to that portion of the active resin yielding a crystalline acetate, M.P. 75°. A good deal of confusion was introduced by later workers, who, although failing to obtain a crystalline acetate, nevertheless applied the name cannabinal to their products. The situation was largely clarified by Cahn [1931], who confirmed the observations of Wood Spivey & Easterfield and established for cannabinal the formula  $C_{21}H_{28}O_2$ . The constitution of cannabinal has been investigated by Cahn [1930-33] and by Bergel [1932], and for it the former proposed structure (I) in which only the positions of the hydroxyl and *n*-amyl groups are to be regarded as uncertain.



In his last communication Cahn states that "cannabinal is not the pharmacologically active principle of *Cannabis indica*" [1933]. It seemed desirable that further investigations on the resin should be made and we have taken up the study of cannabis resin with a view to isolating the substance or substances responsible for its pharmacological action.

The starting point in these investigations was material which corresponded to the high-boiling resin (B.P. 265°/20 mm.) first described by Wood Spivey & Easterfield [1896]. It was found that cannabinal can be removed almost quantitatively as its crystalline *p*-nitrobenzoate (amounting to 25 % of the whole) on



*p*-nitrobenzoylating the resin. On hydrolysis the crystalline ester yields cannabinol as a colourless oil, which gives the above-mentioned crystalline acetate (M.P. 75°) on acetylation. The non-crystalline portion of the *p*-nitrobenzoylated resin gave on hydrolysis a colourless oil from which no crystalline acetate could be obtained.

Active hashish preparations induce a characteristic cataleptic condition in dogs, but this effect, described first by Fraenkel [1903] is not readily made the basis of quantitative assay. Gayer [1928] showed that in various animals—e.g. cats, rabbits, mice—hashish preparations induce corneal anaesthesia and that this effect is characteristic of active fractions of the resin. This Gayer test has been developed by Marx & Eckhardt [1933] using rabbits and has been employed throughout the work described in this paper. The pure cannabinol prepared from the crystalline *p*-nitrobenzoate was found to be highly toxic when injected intravenously into rabbits but, unlike the original resin, did not produce corneal anaesthesia. The cannabinol-free resin on the other hand was much less toxic and always induced corneal anaesthesia in rabbits. This distinction is further emphasized by the fact that an acetone solution of cannabinol was found to be non-toxic after standing for 3 days in contact with air while only slight loss of activity occurred on similar treatment of the cannabinol-free resin.

Attempts to fractionate further the cannabinol-free resin by distillation or crystallization of derivatives failed completely but chromatographic analysis gave promising results. Using activated aluminium oxide as adsorbent it was possible to obtain fairly readily an oil having considerably greater activity in the Gayer test than the starting material. Even better results were obtained by submitting the oily *p*-nitrobenzoate mixture left after separating the cannabinol *p*-nitrobenzoate to chromatographic analysis on activated aluminium oxide. By this procedure remaining traces of cannabinol were removed and an oil was obtained yielding on hydrolysis a product giving a positive result in the Gayer test at a dose of 0.25 mg. per kg. body weight; this material has low toxicity as compared with cannabinol and possesses none of the convulsant action of the latter substance.

Further investigation of this highly active material is in progress and the results will be reported later. We have not as yet obtained any crystalline derivative from the most active preparation and suspect that it does not yet represent the homogeneous active principle.

## EXPERIMENTAL

*Starting material.* The starting material was the resin (B.P. 185–190°/0.6 mm.) obtained on working up hashish of Indian origin in the manner described by Bergel [1930]. The resin was nearly colourless when freshly distilled. It corresponds to the “crude cannabinol” of Wood Spivey & Easterfield and possessed the characteristic pharmacological properties of the original extract.

*Isolation of cannabinol.* The above resin (25 g.) was dissolved in pyridine (110 ml.) and *p*-nitrobenzoyl chloride (36 g.) added. The mixture was refluxed for 4 hr., then poured on a mixture of ice and sufficient H<sub>2</sub>SO<sub>4</sub> to make the resulting suspension acid to Congo red. The precipitate was collected, washed with water, dried and refluxed with light petroleum (750 ml., B.P. 80–100°) for 1 hr. and filtered hot, the filter residue being treated in the same way with a further quantity of light petroleum (250 ml.). The combined filtrates were washed with aqueous Na<sub>2</sub>CO<sub>3</sub>, dried and concentrated to about 150 ml. On standing *cannabinol p*-nitrobenzoate separated. Recrystallized first from alcohol

then from light petroleum (B.P. 80–100°) it formed pale yellow needles m.p. 160° (yield, ca. 6.5 g.). (Found: C, 73.0; H, 6.2; N, 3.2%.  $C_{28}H_{29}O_5N$  requires C, 73.2; H, 6.3; N, 3.2%.) Since cannabinal *p*-nitrobenzoate is very sparingly soluble in methyl alcohol the oil left on evaporating the original light petroleum mother liquors may be largely freed of cannabinal by fractionation with this solvent.

*Cannabinal p-nitrobenzoate.* Cannabinal *p*-nitrobenzoate (1 g.) dissolved in alcohol (80 ml.) was hydrogenated using a platinum oxide catalyst. Absorption of hydrogen ceased when 200 ml. had been absorbed (theoretical 190 ml.). The resulting *p*-aminobenzoate crystallized from methyl alcohol in colourless needles m.p. 149–150°. (Found: C, 78.2; H, 7.4%.  $C_{28}H_{31}O_3N$  requires C, 78.3; H, 7.2%.)

*Cannabinal.* Cannabinal *p*-nitrobenzoate hydrolysed by refluxing with methyl alcoholic KOH (5%) during 1½ hr. gave cannabinal as an almost colourless oil which, with acetic anhydride-pyridine gave in quantitative yield a crystalline acetate m.p. 75° not depressed on admixture with a sample of cannabinal acetate kindly supplied by Dr R. S. Cahn.

#### *Pharmacological tests*

The Gayer test was carried out on rabbits as described by Marx & Eckhardt [1933], the substances being injected in acetone solution (0.5% wt./vol.).

(a) *Cannabinal.* Pure cannabinal prepared by hydrolysing the *p*-nitrobenzoate and subsequent distillation in a high vacuum was used. In doses less than 2 mg. per kg. body weight the material had no visible effect and the corneal reflex remained normal. At any higher dosage the following sequence of events was observed. For about 1 min. the animal behaved normally, but at the end of this time it lay down and in a few seconds rolled over on its side and became rigid, the corneal reflex remaining normal. In any time from a few seconds to 1 min. later, depending on the size of the dose, the rabbit went into violent convulsions terminating in death within about 30 sec.

(b) *Material from non-crystalline p-nitrobenzoates.* After separation of the crystalline *p*-nitrobenzoate the mixture of oily esters from the resin was hydrolysed and the product distilled in a high vacuum. The nearly colourless oil obtained had no effect when injected into rabbits in doses less than 1 mg. per kg. body weight. In a dose of 1 mg. per kg. body weight the drug had no effect for about 3 min., after which time the animal's head began to nod gently and it subsided to its normal sleeping position and remained so. During this period the corneal reflex slowly disappeared until no response could be elicited. The animal could be roused for a few seconds by violent shaking but when left undisturbed quickly relapsed. In doses up to 5 mg. per kg. body weight exactly the same effect was observed, the period of sleep or stupor extending from 30 min. up to 6 or 7 hr. after which the rabbit recovered completely. In doses of 5 mg. or more the animal, although giving a positive Gayer test, died with convulsions within the first 2 hr. after injection. The death after convulsions was presumably due to the presence of some cannabinal in the injected material (cf. below).

#### *Chromatographic analysis of hydrolysate of non-crystalline p-nitrobenzoates*

A sample of the oil (2 g.) prepared by hydrolysis of the non-crystalline fraction of the *p*-nitrobenzoylated resin and subsequent distillation in a high vacuum was dissolved in light petroleum (300 ml.; B.P. 60–80°) and allowed to percolate through a column of activated aluminium oxide (Merck), the chromatogram being developed first with light petroleum (750 ml.; B.P. 60–80°), then with a mixture (1 litre) of equal parts of light petroleum (B.P. 60–80°) and ether.

When the column was viewed in ultraviolet light four distinct bands were visible; from the top downwards these were: (1) yellow 6 cm., (2) colourless 8 cm., (3) yellow 8 cm., (4) blue fluorescent 6 cm. On elution with a mixture of ether and methyl alcohol (4:1) the oils from sections (1), (2) and (3) were found to be inactive when tested on rabbits while that from section (4) gave a positive Gayer test in a dose of 3 mg. per kg. body weight.

The oil from section (4) was therefore combined with that obtained by evaporating the filtrate from the chromatogram, dissolved in light petroleum (B.P. 60–80°) containing 5% ether and re-adsorbed on a column (2 × 30 cm.) of activated aluminium oxide (Merck), the chromatogram being developed with the same solvent mixture. From the top downwards the column showed in ultraviolet light the following bands: (1) purple 3 cm., (2) yellowish blue 10 cm., (3) deep blue 10 cm., (4) strongly blue fluorescent 2 cm., (5) yellow 3 cm. Tests on rabbits showed that the oil from section (4) (50 mg.) was lethal in a dose of 2.5 mg. per kg. body weight and had the typical action of cannabinal. The oil from section (2) (60 mg.) was active in the Gayer test in a dose of 1 mg. per kg. body weight and when injected in a dose of 5 mg. per kg. body weight the animal survived for 10 hr. without any trace of muscular rigidity and died in sleep without any convulsions. The oil from section (3), like the starting material, possessed both convulsant and sleep-producing properties.

*Chromatographic analysis of non-crystalline p-nitrobenzoates.* The oily residue (8 g.) left on removing the crystalline cannabinal derivative from the *p*-nitrobenzoylation product of the distilled resin, was dissolved in light petroleum (B.P. 40–60°) and subjected to adsorption on a column (5 × 45 cm.) of activated aluminium oxide (Merck) previously washed with a solution of phenol in light petroleum to reduce alkalinity. After developing with light petroleum (4 litres) the chromatogram showed six distinct bands when viewed in ultra-violet light. Each of these was separately eluted, hydrolysed, distilled and tested, the filtrate from the column being evaporated and the residue similarly treated and tested. The following table shows the results obtained, the bands in the chromatogram being numbered from the top downwards.

	Colour	Length cm.	Wt. of eluate g.	Millon test	Gayer test	M.L.D. mg.
1	Yellow	3	0.3	—	—	—
2	Colourless	15	1.2	—	—	—
3	Bright blue	3	0.25	+	—	6
4	Colourless	10	2.0	+	—	5.5
5	Yellow	5	0.9	—	—	—
6	Colourless	10	1.2	+	+(2 mg.)	9
7	Colourless	Filtrate	1.5	+	+(0.25 mg.)	5

The biological test results were reproducible in different animals, there being very little variation either in the minimum active dose or in the minimum lethal dose (M.L.D.). Sections (3) and (4) possessed the typical convulsion-producing properties of cannabinal while sections (6) and (7) had no such action. It is clear then from the table that the remaining traces of cannabinal were concentrated in sections (3) and (4), while the material producing corneal anaesthesia was concentrated in sections (6) and (7). The oil from sections (6) and (7) gave, like cannabinal, a precipitate with Millon's reagent.

*Relative stabilities of cannabinal and resin from fraction 7 (above).* A solution (0.5%) of cannabinal in acetone was exposed to the air for 3 days. When tested on rabbits at the end of this time it was found to be non-toxic. A solution of the

resin from fraction 7 (above) after standing for 6 months under the same conditions retained about 25% of its activity in the Gayer test.

#### SUMMARY

*p*-Nitrobenzoylation of the high-boiling pharmacologically active resin from the female flowers of *Cannabis indica* yields crystalline cannabinol *p*-nitrobenzoate and a mixture of resinous esters. Cannabinol is highly toxic and gives a completely negative reaction in the Gayer hashish test on rabbits, while the hydrolysis product of the resinous esters gives a strong positive reaction and is less toxic than cannabinol. The material giving a positive Gayer test has been fractionated by adsorption methods and a product obtained showing a positive Gayer test in rabbits in a dose of 0.25 mg. per kg. body weight.

One of the authors (F. B.) desires to record his appreciation of the hospitality accorded him in the University College and the Laboratory of the Public Analyst, Colombo, and to thank the Director of the Royal Botanic Gardens, Peradeniya, Ceylon, for his generous assistance.

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# XV. THE RELATIONSHIP OF CARBOHYDRATE METABOLISM TO PROTEIN METABOLISM

## I. THE ROLES OF TOTAL DIETARY CARBOHYDRATE AND OF SURFEIT CARBOHYDRATE IN PROTEIN METABOLISM

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EARLIER observations showed that the provision of excess of energy in the human diet is attended by a storage of N and S, the storage being more marked when the surfeit is in the form of carbohydrate than when in the form of fat [Cuthbertson & Munro, 1937]. Simultaneously with these observations, it was demonstrated by Larson & Chaikoff [1937] in one-day superimposition experiments on dogs, that this storage was only provoked by carbohydrate ingested within 4 hr. before or after the protein meal. Moreover, the closer the proximity in time of the surfeit carbohydrate to the meal, the greater was the quantity of N stored.

These effects of surfeit carbohydrate on protein metabolism prompted us to investigate the function of the total carbohydrate of the ordinary adequate diet, as it affects N metabolism, especially with regard to the relative times of ingestion of the protein and the carbohydrate. The present investigation of protein metabolism is divided into four sections:

(I) The effect of complete separation as regards time of ingestion of all, or practically all, the carbohydrate and the protein of an adequate diet (Exps. 1-5).

(II) The effect of separation of only a part of the protein from the carbohydrate in such a diet (Exp. 6).

(III) The effect of taking surfeit carbohydrate at a time separated from that of the ingestion of the protein (Exp. 7).

(IV) The effect on N balance of varying the number of meals in a fixed diet (Exp. 8). (This is intended as a control for certain aspects of the other experiments.)

### EXPERIMENTAL

The analytical procedures were similar to those referred to in a previous paper [Cuthbertson & Munro, 1937]. The fluid intake was kept as constant as possible throughout these experiments.

#### I. *Separate ingestion of dietary protein and carbohydrate*

*Exps. 1 and 2.* In preliminary experiments, two adults, A. H. S. and H. B. C. (Table I), were put on diets adequate for maintenance, containing 80 g. protein and 2740 cal. in the case of the former, and 97 g. and 3400 cal. in the case of the latter. During Period I this food was divided for both subjects into four equal mixed meals, taken at 8.30 a.m., 1 p.m., 4.30 p.m. and 10 p.m. respectively (Tables II and III). Their body-weights remained constant under this regime.

Table I. *The experimental subjects*

Subject	Age yr.	Height cm.	Wt. kg.	Exp. no.	Diet			
					Protein g.	Carbo- hydrate g.	Fat g.	Calories
A. H. S.	30	168	64	1	80	367	101	2740
H. B. C.	24	184	80	2	97	455	127	3400
H. N. M.	22	164	61.5	3	84	400	61	2500
			61.25	6	116	390	87	2880
			64.4	7	76	320	140	2930
			63.9	8	76	320	140	2930
D. P. C.	38	184	83	4	66	408	172	3500
			83.7	5	76	408	174	3560

Moreover, if one allows 1.3 g. as covering the daily faecal and cutaneous losses of N (which were not estimated), the average daily urinary N values for this period (10.82 g. for A. H. S.; 14.27 g. for H. B. C.) agree well with their N intakes of 12.56 and 15.5 g. respectively, confirming the attainment of N equilibrium in each subject.

In Period II, this arrangement of meals was altered so that all the protein and a part of the fat of the diet were divided between the meals at 8.30 a.m. and 4.30 p.m., while the remainder of the fat and practically all the carbohydrate were consumed at 1 p.m. and 10 p.m. (Tables II and III). This almost complete separation of protein from carbohydrate resulted in a marked loss of N, which obtained throughout the period of observation and amounted on the average to 1.77 g. daily for A. H. S. and 2.02 g. for H. B. C. (Figs. 1 and 2). It will be noticed that during no part of the day was there any lack of energy-yielding food material (Tables II and III).

In the case of H. B. C. a return to the previous dietary was made in Period III, and this effected an immediate cessation of the N loss. Under the arrangement of meals in Period II the protein-containing meals were reduced in number from 4 to 2 (Tables II and III); that this procedure was not of itself productive of the marked negative N balance will be subsequently demonstrated in Exp. 8.

*Exp. 3.* This experiment followed along the lines of the previous two, but differed in that the procedure was reversed; the daily urinary collection was separated into two fractions, from 8 a.m.–8 p.m. and 8 p.m.–8 a.m., and account was taken of urea, preformed sulphate and creatinine as well as N excretions. The subject H. N. M. (Table I) partook of a diet containing egg-white, cheese, casein, arrowroot, sugar, butter and apples (84 g. protein, 61 g. fat and 400 g. carbohydrate, in all, some 2500 cal.).

In Period I, all the protein and part of the fat were taken at 9.30 a.m. and 12.45 p.m., while the rest of the fat and all the carbohydrate were spread between 4.30 and 7.30 p.m. (Table IV). During this period of separation of carbohydrate from protein, the daily N intake was 13.36 g., while the urinary output averaged 14.80 g. (Fig. 3), a considerable loss.

In Period II, the carbohydrate and fat of the previous two later meals were taken instead along with the protein and fat at 9.30 a.m. and 12.45 p.m.; the hours of protein ingestion were thus unchanged from Period I, whilst the carbohydrate was now associated with the protein. This produced an immediate fall in urinary N output, down to an average over the period of 12.67 g. (intake 13.36 g.), which is presumably only slightly in excess of N equilibrium. The difference in N output between Periods I and II amounted to 2.13 g. daily.

Table II. *Dietary of subject A. H. S., Exp. 1*

	Period I				Period II			
	8.20 a.m.	1 p.m.	4.30 p.m.	10 p.m.	8.30 a.m.	1 p.m.	4.30 p.m.	10 p.m.
	Same food at all meals (g.)				g.	g.	g.	g.
Lean beef			71.25		142.5	—	142.5	—
Egg			37.5		75	—	75	—
Lettuce			10		20	—	20	—
Apple			50		100	—	100	—
Butter			15		—	30	—	30
Arrowroot			35		—	70	—	70
Sugar			56		—	112	—	112
Calorie distribution at each meal:								
Protein			81		166	—	166	—
Carbohydrate			367		46	688	46	688
Fat			236		240	232	240	232

Table III. *Dietary of subject H. B. C., Exp. 2*

	Periods I and III				Period II			
	8.30 a.m.	1 p.m.	4.30 p.m.	10 p.m.	8.30 a.m.	1 p.m.	4.30 p.m.	10 p.m.
	Same food at all meals (g.)				g.	g.	g.	g.
Lean beef			85		170	—	170	—
Egg			50		100	—	100	—
Lettuce			10		20	—	20	—
Apple			50		100	—	100	—
Butter			15		—	30	—	30
Arrowroot			42		—	84	—	84
Sugar			71		—	142	—	142
Calorie distribution at each meal:								
Protein			100		200	—	200	—
Carbohydrate			455		46	864	46	864
Fat			296		360	232	360	232

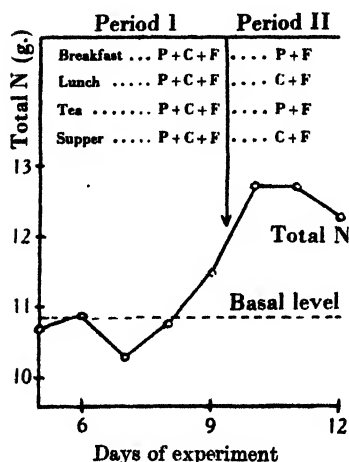


Fig. 1. Exp. 1. Subject A.H.S.

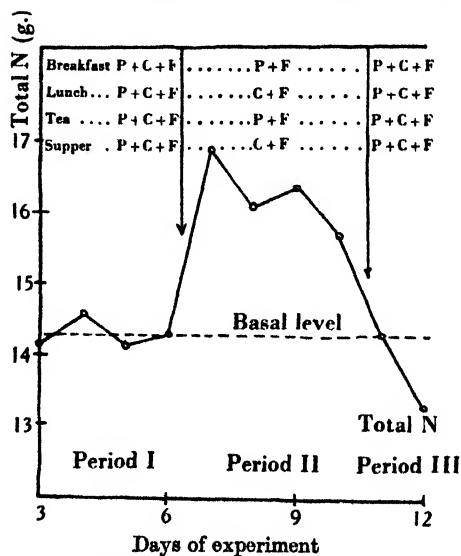


Fig. 2. Exp. 2. Subject H.B.C.

Figs. 1 and 2. Effect on urinary N excretion of separately ingesting the total protein and the carbohydrate of an adequate diet. P=protein. C=carbohydrate. F=fat.

Table IV. *Dietary of subject H. N. M., Exp. 3*

	Period I				Period II	
	9.30 a.m. cal.	12.45 p.m. cal.	4.30 p.m. cal.	7.30 p.m. cal.	9.30 a.m. cal.	12.45 p.m. cal.
Protein	172	172	—	—	172	172
Carbohydrate	—	—	820	820	820	820
Fat	116	116	168	168	284	284

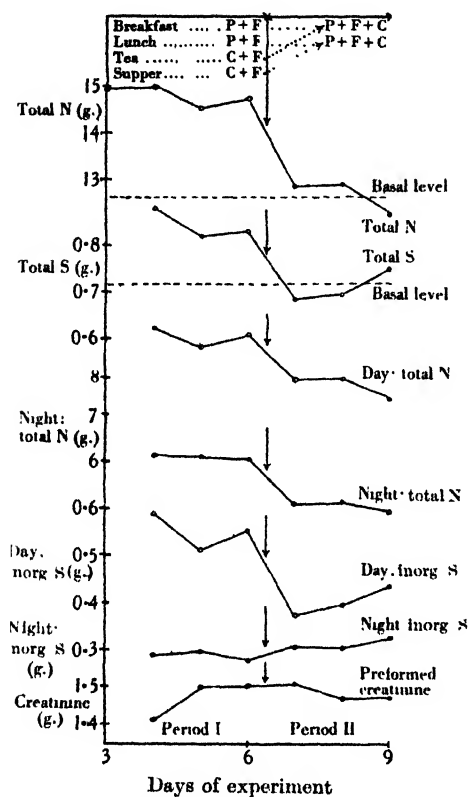


Fig. 3. Exp. 3. Subject H.N.M.

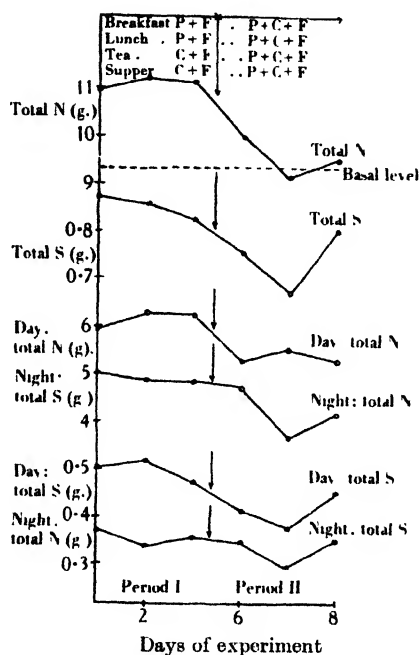


Fig. 4. Exp. 4. Subject D.P.C.

Figs. 3 and 4. Effect of separately ingesting the protein and carbohydrate of an adequate diet.

Urea and also  $\text{NH}_3$  excretion closely paralleled that of total N. The preformed sulphate-S also took the same trend as the N, the excretion in Period I in excess of that in Period II averaging 0.129 g. S daily. The creatinine N output, however, remained relatively constant throughout. A study of the day (8 a.m.–8 p.m.) and night (8 p.m.–8 a.m.) urines indicates that the N content of each was equally affected by the change-over from Period I to Period II. On the other hand, only the day excretion of preformed sulphate was affected, the night excretion remaining relatively constant. It was not possible to record body-weights during the whole course of this experiment.

*Exp. 4.* This was confirmatory of the previous experiment. The subject D. P. C. (Table I) took a diet (Table V) containing 66 g. protein and 3500 calories,



which were so distributed as to enable him to eat meals consisting only of protein and fat at 8.10 a.m. and 1.15 p.m., the meals at 4.45 p.m. and 10.30 p.m. containing only carbohydrate and fat. During Period I the average daily urinary N output was 11.07 g. (Fig. 4), which with a faecal N of 1.29 g. daily (derived from Exp. 5) indicates a marked loss when compared with the intake of 10.52 g. N.

Table V. *Dietary of subject D. P. C., Exp. 4*

	Period I				Period II			
	8.10 a.m. g.	1.15 p.m. g.	4.45 p.m. g.	10.30 p.m. g.	8.10 a.m. g.	1.15 p.m. g.	4.45 p.m. g.	10.30 p.m. g.
Cheese	60	60	—	—	60	60	—	—
Butter	20	20	35	35	50	50	5	5
Egg	125	150	—	—	125	150	—	—
Lettuce	25	—	25	25	25	50	—	—
Apple	—	—	100	100	—	—	100	100
"Oxo"	—	$\frac{1}{2}$ cube	—	—	—	$\frac{1}{2}$ cube	—	—
Sugar	—	—	146	146	48	48	50	50
Arrowroot	—	—	60	60	60	60	60	60

Calorie distribution at each meal:

Protein	130	140	—	—	130	140	—	—
Carbohydrate	—	—	816	816	384	384	432	432
Fat	493	521	293	293	493	521	38	38

The association in Period II of a considerable portion of the carbohydrate of the evening meals with the protein-containing meals of the earlier part of the day (Table V) resulted in a sharp drop in the N output to an average value of 9.27 g. daily on the last 2 days of this period (Fig. 4). N equilibrium was virtually obtained by this normal distribution of the food. The division of the urine into day (8 a.m.—8 p.m.) and night (8 p.m.—8 a.m.) portions showed that the fall in N was equally distributed between them. The total S output took a similar trend to the total N, the major change in it occurring in the day, however. These results are confirmatory of the findings in Exp. 3.

*Exp. 5.* A further experiment was also performed on D. P. C. (Table I), its main object being to determine whether this effect of carbohydrate in promoting the proper utilization of dietary protein were of a quantitative nature or not. A subsidiary consideration was the observation of the changes in body-weight over more prolonged periods of time than the previous separation experiments permitted, and under more uniform conditions.

The diet was similar to that of the preceding experiment (except that 25 g. diabetic bread was included) and contained some 3560 cals. and 76 g. protein; it was so disposed that protein and fat were the constituents of the meals at 8.10 a.m. and 1.15 p.m., and carbohydrate and fat of meals at 4.45 p.m. and 10.30 p.m. On this diet the urinary N averaged 12.80 g. for the 4th and 5th days of Period I (Table VI), the faecal N being 1.29 g. daily. If one allows 0.3 g. for cutaneous loss of N, there is thus a considerable negative N balance over the intake of 12.17 g. N.

On the 6th and 7th days (Period II) 25 g. cane sugar were transferred from the latter two meals of the day and associated with the protein meals at 8.10 a.m. and 1.15 p.m. (half to each meal). A definite reduction in the urinary output, averaging 0.91 g. N and 0.085 g. S daily, took place. In Period III a return to the regime of Period I produced an average daily urinary N over 4 days of 12.58 g.; excretion of S also rose. The transfer of 25 g. cane sugar was again made in

Table VI. *Exp. 5, subject D. P. C. The effect of separately ingesting the protein and carbohydrate in the diet and the effect of varying the amount of carbohydrate associated with the protein*

Period	Days of exp.	Body wt. kg.	Vol. ml.	Urine					
				N (g.)			S (g.)		
				Total	Day	Night	Total	Day	Night
I	1	83.70	—	—	—	—	—	—	—
	2	83.70	—	—	—	—	—	—	—
	3	83.68	2150	13.08	—	—	1.006	—	—
	4	83.68	2090	12.81	7.42	5.39	1.005	0.576	0.429
	5	83.50	1770	12.78	7.65	5.13	1.005	0.612	0.393
II	6	83.20	2210	12.02	6.84	5.17	0.952	0.589	0.363
	7	83.40	1970	11.76	6.57	5.185	0.889	0.532	0.357
III	8	83.50	2290	12.57	7.00	5.57	0.957	0.572	0.385
	9	82.95	2005	12.63	7.56	5.07	0.949	0.577	0.372
	10	82.85	2220	12.38	6.79	5.59	0.991	0.561	0.430
	11	82.80	1965	12.73	7.41	5.32	0.965	0.601	0.364
IV	12	82.75	2280	11.70	6.47	5.23	0.936	0.534	0.402
	13	82.85	2065	12.68	6.51	6.17	0.984	0.529	0.455
V	14	82.73	2180	12.58	7.11	5.47	0.917	0.512	0.405
VI	15	82.72	2270	11.81	7.14	4.67	0.893	0.558	0.335
	16	82.40	2300	11.79	6.72	5.07	0.861	0.488	0.373
	17	82.56	2200	11.54	6.72	4.82	0.893	0.522	0.371
VII	18	82.20	2575	11.71	6.63	5.08	0.861	0.504	0.357
	19	82.65	1770*	11.57	6.46	5.11	0.794	0.422	0.372
	20	82.67	2550	10.78	6.04	4.74	0.840	0.424	0.416

\* Accidental loss of part of day urine. Figures inserted have been arrived at by analysis of the residual urine for N, S and creatinine and then by a calculation based on the constancy of the creatinine excretion in these experiments.

#### Diet

Period I. Meal 1 P + F 8.10 a.m. Meal 2 P + F 1.15 p.m. Meal 3 C + F 4.45 p.m. Meal 4 C + F 10.30 p.m.

Period II, as above, but 12.5 g. cane sugar added to meals 1 and 2 and removed from 3 and 4.

Period III, similar to I.

Period IV, as for I, but 25 g. cane sugar added to meal 2 and removed equally from 3 and 4.

Period V, similar to Period I.

Period VI, as for I, but 100 g. cane sugar added to meal 2 and removed equally from 3 and 4.

Period VII. Days 18 and 19 as for Period VI, but half cane sugar replaced by starch. In addition

100 g. carbohydrate were removed from meals 3 and 4 and taken at meal 1 as cane sugar.

Meals 3 and 4 contained fat and equal amounts of starch and sugar. Day 20, as for preceding 2 days, but half sugar at breakfast replaced by starch.

Period IV, but on this occasion the depression was more transitory than in Period II.

In Period V a single day return to the dietary of Period I was made. Then in Period VI, 100 g. carbohydrate (as cane sugar for the first 2 days) were transferred from the evening meals to the protein meal at 1.15 p.m.; the resulting fall in N and S excretions averaged 0.87 g. and 0.084 g. respectively below the mean of Period III. On the last day of the period starch was substituted for half of the cane sugar, but this did not appreciably affect the N and S excretions.

During the last 3 days of the experiment further slight changes were made in the distribution of the diet, as indicated in Table VI. In the end, loss of body-weight was stopped and N equilibrium was reached at a level 2.02 g. lower than the original level of Period I, and 1.80 g. lower than the intermediate value of Period III. The S excretion also decreased during the final period; its unsteady nature, however, prohibited calculation of the exact amount of the fall. In this experiment the main changes in N and S were confined to the day urines.

II. *Partial separation of protein from carbohydrate*

*Exp. 6.* The possibility that separation of a part only of the protein from the dietary carbohydrate might also result in a loss of N from the body is considered in this experiment. The subject H. N. M. (Table I) was put on a diet containing some 2880 cal. and 116 g. protein, arranged (Table VII) so that he partook in Period 1 of mixed meals (each containing 2.32 g. N) at 9.30 a.m. and 8 p.m.; along with these meals a protein-free jelly of arrowroot and sugar (480 cal. per meal) was consumed. In the intervening hours of the day, 200 g. lean beef were taken at 12.45 p.m. and at 4.30 p.m. respectively. Thus during this period, some 75 % (14.2 g.) of the dietary N was unassociated with carbohydrate; the average daily urinary excretion was 16.57 g. N, representing a slight storage with the intake of 18.8 g. N, if an allowance of 1.3 g. N for faecal and cutaneous losses is made (Table VIII).

Table VII. *Dietary of subject H. N. M., Exp. 6*

	Period I				Period II			
	9.30 a.m. g.	12.45 p.m. g.	4.30 p.m. g.	8 p.m. g.	9.30 a.m. g.	12.45 p.m. g.	4.30 p.m. g.	8 p.m. g.
White bread	81	—	—	81	81	—	—	81
Brown bread	42.5	—	—	42.5	42.5	—	—	42.5
Cheese	3.75	—	—	3.75	3.75	—	—	3.75
Bran	3.75	—	—	3.75	3.75	—	—	3.75
Jam	25	—	—	25	25	—	—	25
Butter	30	—	—	30	30	—	—	30
Apple	50	—	—	50	50	—	—	50
Lean beef	—	200	200	—	—	200	200	—
Arrowroot	45	—	—	45	—	45	45	—
Sugar	75	—	—	75	—	75	75	—
Total N	2.32	7.10	7.10	2.32	2.32	7.10	7.10	2.32
Carbohydrate	195	—	—	195	75	120	120	75

Table VIII. *Exp. 6, subject H. N. M. Partial separation of protein from carbohydrate*

Day of exp.	Diet	Body wt. kg.	Total urinary N output (g.)
2	Period I (association of 25% of the protein with carbohydrate)	61.35	16.39
3		61.15	16.54
4		61.26	17.19
5		61.20	16.19
6		61.25	16.21
7		61.10	16.88
	Mean	61.05	16.57
8	Period II (association of 100% of the protein with carbohydrate)	60.80	16.65
9		60.90	16.20
10		60.95	16.47
11		60.85	16.34
	Mean	60.90	16.42

In Period II the jelly of arrowroot was transferred to the meat meals at 12.45 p.m. and 4.30 p.m. The average daily output during this period was 16.42 g. N. Thus it may be said that, within the limits of our experiment, variation in the quantity of protein unassociated with carbohydrate in a diet does not affect the N balance, amounts as low as 4.64 g. N being sufficient, when ingested along with carbohydrate, to maintain N equilibrium.

III. *Separation of surfeit carbohydrate from dietary protein*

*Exp. 7.* This portion of the work was undertaken to investigate the function of carbohydrate eaten in excess of energy requirements under conditions similar

Table IX. *Dietary of subject H. N. M., Exps. 7 and 8*

	9.30 a.m.	12.45 p.m.	4.30 p.m.	7.15 p.m.
	g.	g.	g.	g.
White bread	50	150	100	25
Brown bread	25	60	60	25
Cheese	—	15	—	—
Bran	—	—	—	15
Apples	—	100	—	100
Butter	—	—	125 g.	—
Jam	—	—	100 g.	—
Milk	—	—	250 ml.	250 ml.

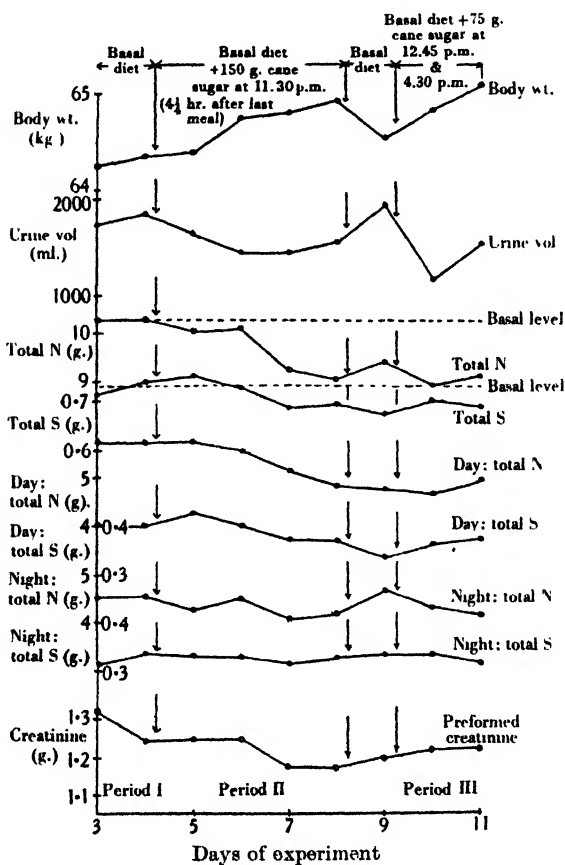


Fig. 5. *Exp. 7. Subject H.N.M. Effect of varying the proximity of a carbohydrate surfeit to the rest of the diet.*

to those in which the total dietary carbohydrate was ingested in the foregoing experiments, i.e. along with and also separate from the dietary protein. It will be remembered that the somewhat similar experiments of Larson & Chaikoff [1937]

were only one-day superimpositions of carbohydrate, and we wished to obtain evidence based on more prolonged surfeit feeding. The subject H. N. M. (Table I) was given a mixed diet (Table IX) containing 76 g. protein and some 2930 cal. In Period I urinary N equilibrium was established at a level of 10.32 g. with an intake of 12.10 g. (Fig. 5).

In Period II 150 g. sucrose were added daily at 11.30 p.m. (4½ hr. after the last meal). For 2 days this surfeit failed to induce any appreciable alteration in N output, but on the 3rd day of surfeit a sharp fall was registered and a new level of 9.14 g. N established. After an interval of one day, on which surfeit was discontinued, Period III was entered on by superimposing the 150 g. sucrose along with the 12.45 p.m. and 4.30 p.m. meals (the surfeit being divided between each) for 2 days. This maintained the N output at an average level of 9.04 g. per day.

An examination of the day (9.30 a.m.–9.30 p.m.) and night (9.30 p.m.–9.30 a.m.) urines shows that both in Period II and in Period III the major portion of the fall in N was attributable to the day fraction, no matter whether the carbohydrate were superimposed in the late evening or in the middle of the day. The fall in N was mainly due to a proportional reduction in urea excretion. In general, total S excretion paralleled total N, any changes noted in the level, however, being confined to the day fraction.

The creatinine output remained relatively constant throughout the course of the experiment. Body-weights were recorded and it will be observed that the giving of surfeit carbohydrate was accompanied by a rise in weight. This rise in weight in no way accounts for the marked and sustained reduction in urinary volume persisting throughout the periods of surfeit; probably this is to be explained by an increase in insensible perspiration, representing an attempt by increased energy metabolism to deal with the surfeit.

#### IV. *N balance in relation to the number of meals*

*Exp. 8.* Since in Exps. 1 and 2 the number of protein-containing meals was reduced from 4 to 2 in the second period of each, it was considered advisable to eliminate this change as a possible cause of the phenomena observed in these experiments (this objection does not apply to Exps. 3 and 4).

Table X. *Exp. 8, subject H. N. M., N balance in relation to the number of meals*

Day of exp.	Diet	Body-wt. kg.	Urine		Faeces	
			Volume ml.	N-output g.	N g.	Fat g.
4	Period I (8 meals per day)	64.05	1895	10.53	Total 3.64	Total 17.08
4		63.50	2238	10.89		
5		63.95	1838	10.83		
7		64.05	1915	10.72		
	Mean	63.89	—	10.74	0.91	4.27
8	Period II (2 meals per day)	63.60	2075	10.74	Total 3.54	Total 17.73
9		63.50	1650	11.83		
10		64.15	1650	11.07		
11		64.00	2155	11.00		
	Mean	63.81	—	11.16	0.89	4.43
12	Period III (8 meals per day)	63.90	2048	10.20	—	—
13		63.95	1960	10.45	—	—
14		—	1990	10.75	—	—

The subject H. N. M. (Table I) was placed on a mixed diet similar to that of Exp. 7 (Table IX), but divided in this case into 8 equal meals separated by intervals of 2 hr. from one another. This gave a very constant N output averaging daily 10.74 g. in the urine and 0.91 g. in the faeces (intake 12.10 g. N). In Period II this diet was compressed into 2 equal meals, taken at 11 a.m. and 5 p.m. respectively. The effect on the N output was an evanescent rise (probably due to the alteration in the times of protein ingestion), the mean for all 4 days of this regime being only 11.16 g. N per day. The faecal fat and faecal N were unaltered by this change. In Period III the 8 meals were restored; this produced a temporary depression of N, followed by a return to the average output in Period I (Table X).

It may accordingly be said that since a change from 8 to 2 meals induces only a slight alteration in daily N output, the reduction of the protein-containing meals from 4 to 2 in Exps. 1 and 2 is at most only a minor factor, if indeed one at all, in the production of the marked losses of N observed therein.

### DISCUSSION

The salient features of our experiments may be grouped under the divisions suggested in the introductory remarks.

I. Exps. 1-4 indicate that the output of N and S from the body exceeds the intake when the adult human subject is fed on a diet abnormal only in one respect, viz. that the ingestion of the protein moiety is dissociated in time from that of the carbohydrate. This loss of N and S from the body occurs immediately on separating protein from carbohydrate and persists throughout the period of separation. In assessing the extent of this loss we have taken the mean daily level of urinary N output during the period of mixed meals as representing N equilibrium and have regarded as N lost from the body any excess output over and above this mean level. On such a basis, during the periods of separation of protein from carbohydrate the mean daily output of urinary N in our 4 experimental subjects was 1.93 g. greater than the N output when on mixed meals, i.e. there was a daily average of 1.93 g. N lost from the body. The individual data for these 4 subjects are appended in Table XI.

Table XI

Exp.	Subject	Material lost daily			Body-wt. kg.	Loss per kg. wt. g. N	Loss per sq. metre surface g. N
		N g.	S g.	N:S ratio			
1	A. H. S.	1.77	—	—	64	0.025	0.94
2	H. B. C.	2.02	—	—	80	0.028	1.04
3	N. N. M.	2.13	0.129†	15.5:1*	61.5	0.035	1.20
4	D. P. C.	1.81	0.118	15.3:1	83.7	0.022	0.89

\* Urea-N: preformed sulphate-S.

† Preformed sulphate-S.

It will be seen that the losses of N vary between 1.77 and 2.13 g. daily for the different subjects. Age may be a factor in determining the extent of the loss; in the present series of experiments there is an inverse relationship (see Table I). However, the variations in individual loss are found to be reduced if calculated per kg. body weight, or per square metre body surface. The relative constancy from individual to individual suggests that the loss is conditioned by the level of the endogenous metabolism; this possibility is substantiated by an examination of the more extensive data given for the endogenous N output, such as those

of Deuel *et al.* [1928], in which the value of 0.0241 g. per kg. body-weight was the daily excretion of urinary N after the subject had lived for more than a month on a protein-free diet—a figure which strikingly resembles the figures given in Table XI.

Is it by mere accident that there is a similarity between the daily amount of endogenous N excretion and the daily loss of N which occurs in these separation experiments, or does it really imply that the separate ingestion of carbohydrate and protein results in an inability of the organism to make good the endogenous loss of tissue protein? It must be remembered that only in Exp. 5 was the separation of carbohydrate and protein maintained over a prolonged period, but in this experiment the evidence is not clear-cut, for the separation was intermittent. We cannot therefore exclude the possibility that this loss of N and S might diminish in the course of prolonged separation, perhaps owing to the development of some compensatory mechanism. Exp. 5 does however clearly indicate that the N loss is accompanied by a decline in body-weight (Table VI). The significance of the other results of this experiment are discussed below.

The possibility of an endogenous source for the N lost is also supported by an examination of the separation of the 24 hr. urines into day and night fractions (Exps. 3 and 4). It is seen that the loss of N during the separation of the two food-stuffs is equally distributed between each fraction. This would apparently indicate a source of N which is unaffected by digestive and absorptive processes, i.e. an endogenous source. If this is so one might anticipate that the output of S would follow the same course, but examination shows that S loss is mainly confined to the day urine (Figs. 3 and 4). It is common knowledge that the metabolism of the S moiety of protein generally occurs more rapidly than that of the N fraction.

As regards the nature of the material lost during separation, Table XI shows that the N:S ratios of this material resemble closely the N:S ratio of muscle-protein (15.5:1), but it must be admitted that the quantities of N and S involved are small. (In Exp. 3, the S estimated was preformed sulphate-S, and accordingly the appropriate ratio is urea-N:preformed sulphate-S.) This finding of an N:S ratio similar to that of muscle is not however proof that the loss was from such a source; the material involved may well have been derived from a storage protein of similar ratio, utilized at the time of separation to replenish the endogenous requirements. It is significant that the creatinine excretion was relatively unaffected during the course of these separation procedures.

II. Exp. 6 indicates that for the normal metabolism of the dietary protein only a small fraction of it requires to be ingested simultaneously with the carbohydrate. In this experiment protein corresponding to only 4.64 g. N out of the 18.8 g. N in the diet was taken with the dietary carbohydrate, and yet the N output was the same as when all the protein (18.8 g. N) was taken with the carbohydrate (Table VIII).

The corollary does not appear to hold, viz. that the association of a small amount of carbohydrate with the protein of the day will necessarily maintain normal protein metabolism and prevent a loss of N (Exp. 5, Table VI).

III. By way of contrast to these N-sparing effects of carbohydrate in an adequate diet, the ingestion of carbohydrate in excess of energy requirements produces an actual storage of N. This is shown mainly in the day fraction of the 24 hr. urine, suggesting that it is the immediate products of digestion which are affected by the surfeit. The excretion of S is similarly depressed, the fall being again confined to the day output (Fig. 5).

It was observed by Larson & Chaikoff [1937], in their one-day superimposition experiments on dogs, that surfeit lost its N-storing effect when applied more than 4 hr. before or after the protein meal. With this we are in agreement, but we would also add that over longer periods than a single day, surfeit carbohydrate ingested separately from protein does eventually exercise a saving effect similar to that of surfeit carbohydrate taken simultaneously with the protein. The distribution of the saved N between the day and night fractions of the 24 hr. urines is identical under both conditions, viz. when the carbohydrate is taken apart from the protein (in the late evening) or along with the protein (in the afternoon) (Fig. 5).

IV. The possibility that the phenomena observed in Exps. 1 and 2 might be due to the change in the number of the protein-containing meals in the course of these experiments is discounted by Exps. 3, 4 and 5, to which this objection does not apply (Figs. 3, 4 and Table VI). It is also negatived by Exp. 8, in which the number of meals of a fixed diet were varied from 8 to 2 without seriously affecting the level of N output (Table X). This is consistent with the results of other workers, as reviewed by Cathcart [1921]. It may be further noted that this change in the number of meals in Exp. 8 did not alter the utilization of the protein and fat, since both faecal N and faecal fat were found to be unaltered in amount by this procedure. It would indeed appear that no appreciable economy in food is effected by eating more frequently than twice a day.

#### *Mechanism of N-sparing and N-saving*

In the foregoing discussion we have presented two separate aspects of carbohydrate in its relation to protein metabolism. These two may be termed N-sparing in the case of the adequate diet (Exps. 1-5), and N-saving in the case of the surfeit diet (Exp. 7). They differ in certain respects.

The separate ingestion of the carbohydrate and the protein of the adequate diet results in a loss of N from the body, and this loss may be spared if the carbohydrate and the protein are ingested together. To produce this sparing effect requires the close approximation of the protein to the carbohydrate (Figs. 1, 2, 3, 4 and Table VI). It occurs at once on ingesting the carbohydrate along with the protein; moreover, the sparing is equally distributed over the day and night excretions of N (Figs. 3 and 4). The N spared is not necessarily proportional to the amount of carbohydrate present along with the protein (Table VI).

The N-saving effect of carbohydrate present in excess of energy requirements differs from the N-sparing effect in that surfeit carbohydrate ingested at any period of the day is capable in the long run of causing this storage of N in the body (Fig. 5), in that it takes several days of surfeit to exert its full effect [Cuthbertson & Munro, 1937] and in that, in contrast to N-sparing, N-saving is mainly effected on the day excretion of N, even if the carbohydrate be taken at night (Fig. 5). The N thus stored in the body is roughly proportional to the amount of surfeit carbohydrate taken.

In seeking to explain the loss of N from the body which results from the separate ingestion of the protein and the carbohydrate in an adequate diet, four hypotheses may be considered. All four assume that the underlying factor is an increased immediate disposal of the food protein, leading to an insufficiency of protein remaining to replace the endogenous N loss.

(a) If an insufficient quantity of energy were present in the protein-fat meals of the separation period, it might make necessary an excessive utilization of food protein for immediate energy purposes; hence, less protein would be left over for tissue needs.



(b) The absence of carbohydrate from the protein-fat meals might have to be made good by production of carbohydrate from food protein, thus lessening the amount of protein available for endogenous requirements.

(c) In the absence of carbohydrate from the protein-fat meals, a more rapid combustion of protein by its own specific dynamic action might occur.

(d) A specific inhibitory action of carbohydrate on the deaminases of the body might exist, an inhibition which would not come into play unless both carbohydrate and protein were simultaneously ingested. This last theory can be made to embrace both N-sparing and N-saving effects.

The first of these hypotheses may be disposed of by reference to Tables II-V, in which it is shown that there is no deficiency of energy in the protein-fat meals of the periods in which carbohydrate and protein were separately ingested. One cannot therefore ascribe the N loss in these experiments to insufficiency at any particular period of the day, unless it be assumed that fat is not metabolized at a sufficiently rapid rate to cover the energy needs immediately after the protein-fat meals of the day.

The second argument seeks to explain the N loss as an attempt to make available the non-N residues of protein metabolism for carbohydrate synthesis. It is true that these residues would tend to compensate for the absence of carbohydrate in the protein-fat meals of the separated diet, but they would also be productive of an excessive deamination of the protein of the diet and a consequent protein-starvation of the tissues. This suggestion would seem unlikely in the face of the adequate carbohydrate available at other periods of the day (Tables II-V). It might of course be further contended that for the complete oxidation of the fat present in the protein-fat meals, newly formed carbohydrate might be required, thereby increasing the demand on the non-N protein moiety. If such be the case, the large variations in the quantity of fat present in the protein meals of our diets might be expected to cause proportionately varying losses of N. Such was not the case, the losses of N being on the contrary reasonably uniform (Table XI).

The third consideration is that, in the absence of freshly ingested carbohydrate, the specific dynamic action of the dietary protein must be met at the expense of the protein itself. That this is unlikely is suggested by a study of the dietaries of Exps. 1-4 (Tables II-V). In the period of separation of protein from carbohydrate in Exps. 1 and 2, the 2 meals of protein were separated from those containing carbohydrate by some 4 hr., whereas in Exps. 3 and 4, the periods of separation of the first protein meal from the first carbohydrate meal were 7 hr. and 8½ hr. respectively. Since specific dynamic action after a protein meal is a prolonged effect [Williams *et al.* 1912], one would expect a commensurately greater loss of N in the second pair of experiments, to say nothing of variations dependent on protein intake, if such losses were actuated by specific dynamic action. Such a view is not tenable in the light of the relative uniformity of our results (Table XI).

We prefer to believe that both N-sparing and N-saving have one underlying mechanism, suggested by the observation of Krebs [1935] that deamination by liver slices is inhibited in the presence of easily oxidizable substances such as lactate, pyruvate, succinic and  $\alpha$ -keto acids (but curiously enough, not glucose). In regard to the N-sparing of the ordinary adequate diet, the presence of carbohydrate (perhaps yielding a more active form of glucose within the body) along with the protein is sufficient to secure that some of the amino-acids escape deamination and go to replace the effete products of endogenous metabolism; N balance is thus attained. However, if all the dietary protein be ingested

separately from the carbohydrate, the deaminases are given a free hand, this sparing effect of carbohydrate no longer obtains and deamination is then complete. Further, to this waste of food N is added the unreplaced endogenous quota, so that there is produced an output of N which exceeds intake by an amount equal to the daily endogenous N.

The N-saving effect of surfeit carbohydrate may possibly be explained by the same mechanism; here, even more amino-acids are spared deamination by the inhibiting action of the greater concentrations of carbohydrate resultant on the surfeit intake. Thus in the blood a plethora of amino-acids is produced, and by the law of mass action the storage depots are further charged with protein. This would account for the finding of Larson & Chaikoff [1937] that, in one-day superimposition experiments, carbohydrate was only effective when ingested within 4 hr. before or after the protein, i.e. when its absorption fell within the main phase of protein metabolism. In more prolonged surfeit feeding (Exp. 7) storage of the carbohydrate would also seem to play a part, for surfeit taken outside the 4 hr. limits does have a N-saving effect in the course of a few days (Fig. 5), which probably represents the (variable) time taken to fill up the carbohydrate stores. The eventual result of charging these stores would be that the freshly ingested carbohydrate of the ordinary daily meals is unable to pass out of circulation with its wonted rapidity; it thus produces a higher concentration of carbohydrate within the organism than it normally would and simulates thereby the effect of giving a large draught of surfeit carbohydrate along with the meal. This would of necessity imply that owing to the prolonged period of surfeit feeding the Staub-Traugott phenomenon would be less marked, indicating a less effective disposal of freshly absorbed carbohydrate. Whatever may be the mechanism involved, carbohydrate ingested apart from protein seems to produce an inhibition of deamination of exactly similar extent and time-distribution to that of surfeit carbohydrate taken along with the protein.

That this egress of the carbohydrate from currency and that the availability of stores for this purpose play an integral part in determining the effect of dietary carbohydrate on protein metabolism seems more certain from the results obtained in Exp. 6. Here 2 meals of an adequate (but not surfeit) diet were loaded with all the dietary carbohydrate (some 390 g.) but yet a similar N balance was obtained as when there was a distribution of the same carbohydrate over all 4 meals of the day. That is, if there is excessive ingestion of carbohydrate at one particular meal at the expense of carbohydrate at other meals of that day, the excessive ingestion is at once counteracted by a legacy (from the carbohydrate-poor meals) of stores depleted in carbohydrate. The fluctuations in concentration of current carbohydrate at the sites of deamination would thus be more or less similar after each meal, even if the dietary carbohydrate were disposed unevenly between the different meals. We are of the belief that such a mechanism is the basis whereby N balance is made possible in the organism on the ordinary adequate diet, subjected as it is to vagaries of time and tide of carbohydrate intake.

This capacity for storage of carbohydrate probably also explains the curious results of Exp. 5 (Table VI), in which varying amounts of carbohydrate were given along with the dietary protein, in an attempt to reduce the loss of N which occurs on separation of carbohydrate from protein in an adequate diet. It was found that 100 g. carbohydrate so given were no more effective than 25 g., the N loss being equally reduced, but not abolished. Only when a larger proportion of the total dietary carbohydrate was transferred to the protein meals did N loss eventually cease to occur. This would seem to indicate that the carbohydrate stores at the times of the protein meals were not replete, but rather depleted, and

hence that 25 g. sugar and 100 g. sugar were equally well disposed of, the larger amounts being however less rapidly dealt with by the carbohydrate stores.

Further evidence of a carbohydrate mechanism in protein metabolism may be adduced from the experiments of Silver [1937] which indicate that the amount of carbohydrate in a diet is a factor in the establishment of N equilibrium on that diet. It was found that, by keeping the protein and energy content similar, but varying the carbohydrate-fat ratios of a series of diets, the N balance was favourably influenced by larger proportions of carbohydrate in the diet. The metabolism of the Esquimaux, and of other races whose dietaries contain very little carbohydrate, must presumably be such that the non-N moieties of the large quantities of protein in these diets are deviated to the filling up of the carbohydrate stores and thus successfully take the place of carbohydrate. In this connexion it is recalled that Voit [1881] showed that N and C equilibrium could be eventually established in dogs fed on a high meat diet in which virtually no carbohydrate was present.

#### SUMMARY

1. It has been demonstrated on 4 human subjects that when the protein and the carbohydrate moieties of an adequate diet are separately ingested over short periods of time, there is a negative N balance of some 2 g. daily (mainly urea).

2. This loss is equally distributed between the day and night portions of the 24-hourly urines. There is a corresponding loss of S which, however, is confined to the day urines. Creatinine excretion is relatively unaffected by this procedure.

3. N metabolism is undisturbed by dissociating a part only of the dietary protein from the carbohydrate; this is in contradistinction to the N loss resultant on complete separation of protein and carbohydrate. The association of a small fraction of the dietary protein with the carbohydrate is sufficient to maintain N equilibrium.

4. Carbohydrate, if ingested over a sufficiently long period in excess of the energy requirements, causes storage of N and S no matter whether the carbohydrate be taken along with, or apart from the dietary protein.

5. The variation of the number of meals in a fixed diet from 8 to 2 causes a transitory loss of N due to the disturbance in time relationships. The digestibility and absorbability of the protein and fat of the diet are apparently unaffected by this procedure.

6. The changes in protein metabolism involved in these experiments and the possible mechanisms of their production are discussed.

In conclusion we wish to express our thanks to Prof. E. P. Cathcart and to Prof. G. M. Wishart for their advice and encouragement. Our thanks are also due to Miss Andross and her colleagues of the College of Domestic Science for their ever-ready help in the preparation of some of these diets. We are indebted to Messrs H. B. Cowan and A. H. Smith for acting as subjects in two of these experiments, and finally to Mr McCutcheon for his help with the analyses.

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## XVI. SOME EFFECTS OF PYROPHOSPHATE ON THE METABOLISM OF TISSUES

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PYROPHOSPHATE has been shown by Dixon & Elliott [1929] to inhibit the respiration of minced tissue. For liver mince the inhibition was less than with cyanide, and small concentrations produced a slight acceleration. With muscle, on the other hand, pyrophosphate produced a greater inhibition than cyanide,  $M/30$  pyrophosphate causing more than 80 % inhibition. According to Keilin [1929] pyrophosphate has no effect on indophenol oxidase (cytochrome oxidase). Dixon & Thurlow (quoted by Dixon & Elliott [1929]) found that succinic dehydrogenase is definitely inhibited by  $M/1000$  pyrophosphate while xanthine dehydrogenase is unaffected by  $M/25$ . More recently, Leloir & Dixon [1937] have made a systematic survey of different dehydrogenases and have found that, of the nine dehydrogenases studied, succinic is the only one which is inhibited by pyrophosphate, so that pyrophosphate probably acts as a respiratory inhibitor by specific inhibition of this enzyme.

Succinic dehydrogenase has come into prominence recently with Szent-Györgyi's theory of tissue respiration [1937] in which succinic, fumaric, malic and oxaloacetic acids, with their appropriate enzyme systems, can act as hydrogen transportases. Succinate is the last member of the chain of reactions and from this H is transferred to molecular  $O_2$  through cytochrome and cytochrome oxidase. If the  $C_4$  dicarboxylic system accounts for the bulk of tissue respiration one would expect respiration to fall off when succinic dehydrogenase is inhibited, the extent, of course, depending on whether or not the concentration of succinic dehydrogenase is the limiting factor. This was indeed shown to be the case by Annau [1935], Gözsy & Szent-Györgyi [1934], Greville [1936], and Stare [1936] who found that malonate inhibited the respiration of various tissues. Boyland & Boyland [1936] reported an inhibition in respiration of tumour tissue by the addition of malonate. Elliott & Greig [1937], using Ringer-Krebs medium, found that malonate inhibited the respiration of liver slices about 15 % and that of kidney slices by 50–60 %, presumably by specific inhibition of succinic dehydrogenase.

Johnson [1936] found that the rate of aerobic removal of Na  $\alpha$ -glycerophosphate by normal minced brain tissue is increased by added Na pyrophosphate. He considers this effect similar to that of adenylyl pyrophosphate acting as coenzyme for yeast glycerophosphate dehydrogenase as shown by Lehmann [1934].

Pyrophosphate seems to exert a stimulating effect on certain other enzymes. Green [1936], working on isolated enzyme systems, found that pyrophosphate has no effect on lactic dehydrogenase but causes 100 % acceleration of malic dehydrogenase.

Peters & Sinclair [1933, 1], using minced pigeon brain in phosphate buffer, found that Na pyrophosphate added alone had little influence upon maintenance of respiration in the surviving tissue but that, added with lactate, it increased the respiration rate considerably. Respiration was also maintained at a more constant rate over a period of 3 hr. instead of falling off rapidly as was the case with added lactate alone. They found that the improvement in respiration by pyrophosphate with added lactate was, in part, due to an increase in fluoride-sensitive respiration, and suggested that the hexosediphosphate (or triose-phosphate) system is in some way involved in the metabolism of lactate. The optimum concentration of pyrophosphate was 0.2% which is of the same order as that possible in the cell. Peters considered that these substances (lactate, pyrophosphate and probably glycerophosphate) may be present in the tissue and cause the initial high rate of respiration, but diffuse out of the cell and gradually become ineffective.

In avitaminous brain Peters *et al.* [1933, 2; 1935] found that pyrophosphate ( $M/100$ ) enhanced the catatorulin effect of vitamin  $B_1$  in the metabolism of lactate and pyruvate. It may be that cocarboxylase is synthesized in this tissue from vitamin  $B_1$  and pyrophosphate.

It thus appears that pyrophosphate has several possible effects on the respiration mechanisms of minced tissues. It can increase oxidations by a stimulating effect on malic or  $\alpha$ -glycerophosphate dehydrogenations and it can increase lactate oxidation and the catatorulin effect of vitamin  $B_1$ , but it can also inhibit respiration through its effect on succinic dehydrogenase. The results of a survey of the effects of pyrophosphate on respiring slices of different tissues are presented here.

#### EXPERIMENTAL

The methods employed were those described by Elliott and co-workers [1934, 1935] for the Dixon-Keilin differential manometer. When glucose was present in the medium its concentration was 0.24%. The concentrations of added substrates were: *dl*-lactate  $M/25$ ; pyruvate, succinate and *l*-malate  $M/50$ . The concentration of pyrophosphate varied as shown in the table. The solution, freshly prepared for each experiment, was brought to pH 7.4 by addition of dilute HCl [see Leloir & Dixon, 1937]. The experimental period was 90 min. in all cases.

The rat tissues were prepared as described by Elliott *et al.* Ox eyes were obtained from the slaughter house on the morning of killing and the retina was removed as will be described in a later paper. With the rat tissues the results were calculated on the final dry wt. [see Elliott *et al.* 1937]. With retina the tissue disintegrated to such an extent, especially after the addition of acid, that final dry wts. were only approximate. The results were therefore calculated on the initial dry wts. obtained from the wet wt. of tissue taken and the dry wt./wet wt. ratio determined on separate samples.

The terms used for expressing results are those defined by Elliott *et al.* Variations of 10% are considered to be within the experimental error and when the apparent effect of pyrophosphate is within this range the % effect is not given in Table I. When the values obtained are small, as is often the case for  $Q_A$ , the effect of pyrophosphate may cause a large % change but the values are too small for accurate experimental measurement. In these cases the % change is not given but when there is a definite acceleration or inhibition it is designated by a plus or a minus sign in Table I.

Table I

Tissue	Addition	Final conc. of pyrophosphate	Glucose	$-Q_{O_2}$	R.Q.	$Q_A$	% effect of pyrophosphate	
							$Q_{O_2}$	$Q_A$
Rat tumour Walker 256	None	-	+	9.2	1.0	+ 20.2		
	Pyrophosphate	$M/100$	+	10.5	0.78	+ 19.0		
	None	-	+	10.1	0.98	+ 18.7		
	Pyrophosphate	$M/30$	+	6.5	0.65	+ 17.7	- 35	
	None	-	-	11.0	0.68	+ 0.9		
	Pyrophosphate	$M/30$	-	10.1	0.69	+ 1.4		
	Lactate	-	-	11.9	0.79	- 0.5		
Rat testis	Lactate + pyrophosphate	$M/50$	-	10.5	0.76	+ 1.1	- 12	+
	None	-	-	6.9	0.68	+ 1.3		
	Pyrophosphate	$M/30$	-	4.8	0.64	+ 2.2	- 30	
	None	-	+	11.5	0.94	+ 6.9		
	Pyrophosphate	$M/30$	+	9.9	0.83	+ 5.0	- 14	-
	Lactate	-	-	16.3	0.86	- 2.8		
	Lactate + pyrophosphate	$M/50$	-	11.9	0.83	1.1	- 27	-
Rat liver	None	-	- *	12.8	0.85	+ 0.1		
	Pyrophosphate	$M/30$	-	11.6	0.76	+ 0.4	- 10	
	Pyrophosphate	$M/30$	+	8.8	0.81	- 2.6	- 31	+
	Lactate	-	-	12.5	0.81	- 3.3		
	Lactate + pyrophosphate	$M/30$	-	9.5	0.88	0.3	- 24	
	None	-	-	12.5	0.75	- 1.3		
	Pyrophosphate	$M/30$	+	10.6	0.72	- 1.8	- 16	
	Pyrophosphate	$M/30$	+	10.0	0.85	+ 1.8	- 20	
	Lactate	-	-	13.7	0.89	- 4.5		
	Lactate + pyrophosphate	$M/30$	-	12.7	0.88	- 0.6		- 88
	None	-	-	16.7	0.93	- 5.8		
	Pyrophosphate	$M/50$	+	15.7	0.96	- 7.4		
Rat brain	Lactate	-	-	11.9	0.95	- 2.8		
	Lactate + pyrophosphate	$M/50$	-	12.1	0.94	- 2.2		
Rat kidney	None	-	+	25.9	0.90	1.1		
	Pyrophosphate	$M/100$	+	27.1	0.79	+ 2.8		+
	None	-	-	24.4	0.75	- 0.6		
	Pyrophosphate	$M/50$	-	22.7	0.74	+ 3.6		+
	None	-	+	27.7	0.84	+ 0.2		
	Pyrophosphate	$M/50$	+	31.4	0.82	+ 3.1	+ 13	+
	None	-	+	25.9	0.80	+ 0.4		
	Pyrophosphate	$M/30$	+	30.2	0.78	- 3.6	+ 17	-
	Pyrophosphate	$M/100$	+	30.7	0.83	+ 2.1	+ 18	+
	Pyrophosphate	$M/1000$	+	26.8	0.89	- 1.6		+
	None	-	+	25.1	0.85	- 0.9		
	NaCl 4M/30	-	+	26.3	0.82	+ 1.6		+
	None	-	-	19.6	0.79	- 1.4		
	NaCl 4M/30	-	-	17.8	0.69	+ 0.4		
	Succinate	-	-	46.7	0.74	- 18.2		
	Succinate + pyrophosphate	$M/50$	-	40.4	0.79	- 7.1	- 12	61
	Malate	-	-	34.3	1.18	- 17.1		
	Malate + pyrophosphate	$M/50$	-	29.7	0.95	- 3.7	- 13	- 78
	Pyruvate	-	-	37.8	1.23	- 20.2		
	Pyruvate + pyrophosphate	-	-	36.2	1.08	- 7.3		60
	Lactate	-	-	27.2	0.81	- 6.5		
	Lactate + pyrophosphate	$M/50$	-	27.0	0.83	- 2.3		- 65

\* The presence or absence of glucose does not affect the normal respiration of liver [see e.g. Elliott *et al.* 1937].

Table I (cont.)

Tissue	Addition	Final conc. of pyro- phosphate	Glucose	$-Q_{O_2}$	R.Q.	$Q_A$	% effect of pyrophosphate	
							$Q_{O_2}$	$Q_A$
Ox retina	None	—	+	9.8	0.86	+ 8.4		
	Pyrophosphate	<i>M</i> /50	+	15.2	0.98	+ 9.9	+ 55	
	None	—	—	7.3	0.99	— 0.9		
	Pyrophosphate	<i>M</i> /50	—	9.3	1.00	— 0.8	+ 27	
	Lactate	—	—	11.1	0.90	— 2.3		
	Lactate + pyro- phosphate	<i>M</i> /50	—	14.5	0.97	— 3.1	+ 31	+
	None	—	—	6.4	0.93	— 1.0		
	Pyrophosphate	<i>M</i> /50	—	7.1	0.89	— 0.3	+	
	Lactate	—	—	11.1	0.85	— 2.3		
	Lactate + pyro- phosphate	<i>M</i> /50	—	16.2	0.95	— 2.9	+ 46	+
	None	—	+	11.6	0.88	+ 7.9		
	Pyrophosphate	<i>M</i> /50	+	15.2	0.91	+ 9.3	+ 31	+ 18

## DISCUSSION

The effect of pyrophosphate on tissue slices varies with the concentration of pyrophosphate, the tissue used and the substrate being metabolized. With rat tumour (Walker 256), dilute pyrophosphate (*M*/100) in the presence of glucose increases the respiration slightly without affecting glycolysis. A more concentrated solution (*M*/30) in the presence of glucose inhibits respiration 35 %. In both cases the R.Q. is considerably decreased. In the absence of glucose, *M*/30 pyrophosphate has practically no effect on respiration and glycolysis. It also has no effect on lactate metabolism. Elliott *et al.* [1935] and Elliott & Greig [1937] showed an extremely low succinoxidase content in Walker 256 tumour so that either respiration does not go through succinate or succinoxidase is likely to be the limiting factor. The definite effects of pyrophosphate, particularly on the R.Q., show that it does penetrate cells to a certain extent. If we can assume that it penetrates considerably we must expect succinoxidase inhibition; the absence of more marked effects on respiration then means that succinate is not an essential intermediate of respiration in this tissue.

With testis, which is also low in succinoxidase activity, pyrophosphate, in a concentration of *M*/30, inhibits  $O_2$  uptake to some extent both in the presence and absence of glucose. Oxidation of lactate is inhibited by 27 % by pyrophosphate indicating that removal of this acid in testis is probably to some extent dependent on the succinate system.

Pyrophosphate has little effect on the normal respiration of liver slices, but in the presence of glucose respiration is inhibited by 31 % and acid formation is increased. With added lactate  $O_2$  uptake is inhibited by about 24 % and acid removal is practically completely inhibited. This indicates that the metabolism of liver, especially with added lactate, is, like that of testis, at least in part dependent on the succinoxidase system.

Pyrophosphate has no effect on the respiration of brain slices in the presence of glucose or on its metabolism of lactate. The fact that our experimental procedure and the duration of our experiment were quite different from those of Peters may explain the absence in our experiment of the stimulating effect of pyrophosphate on lactate metabolism. Since pyrophosphate does not inhibit

lactate oxidation in brain slices, this would seem to indicate that the main path of oxidation is not through succinate. However, the absence of any effect whatever might indicate that pyrophosphate does not penetrate the cells of sliced brain.

With kidney the effects are different from those found with the above tissues. In the absence of glucose pyrophosphate causes a slight inhibition in  $O_2$  uptake and some accumulation of acid; in the presence of glucose there is increased respiration and increased glycolysis. These results may indicate that there is an alternative path of oxidation to that proceeding by way of succinate, or it may be that succinoxidase is not completely inhibited and perhaps other systems such as the malic and lactic systems are accelerated so that the net result is increased oxidation in the presence of glucose.

That the effect of pyrophosphate on glucose oxidation is not a salt effect was shown by adding NaCl approximately isotonic with the pyrophosphate used. This had no effect on respiration but it caused a small increase in acid formation.

With added succinate the effect of pyrophosphate is similar to that of malonate [Elliott & Greig, 1937]. The removal of acid groups is inhibited 60 % and  $O_2$  uptake is inhibited about 12 %, but respiration is still greater than that in the absence of substrate or inhibitor, indicating that some succinate is still being oxidized. This is to be expected in view of the extremely high succinoxidase content of kidney [see Elliott and Greig, 1937]. In the presence of pyruvate, pyrophosphate inhibits removal of acid groups about 60 %, i.e. about the same as for succinate, but the  $O_2$  uptake remains the same as without inhibitor. Pyrophosphate decreases acid disappearance in the presence of malate by about 78 % and respiration is decreased to about the same extent as with added succinate, although it is still higher than in the absence of substrate. These results are similar to those of Elliott and Greig with malonate and indicate that the succinoxidase system is concerned with the complete oxidation of malate to  $CO_2$  and  $H_2O$ . With added lactate, pyrophosphate has no effect on  $O_2$  uptake but decreases acid removal by 65 % which also indicates the participation of the succinoxidase system in the complete oxidation of this substrate.

Ox retina differs from the other tissues studied in its reaction to pyrophosphate. Like testis it has a low succinoxidase content but added pyrophosphate, instead of inhibiting respiration, causes a large increase in  $O_2$  uptake and a small increase in glycolysis in the presence of glucose. Without added substrate there is usually some increase in respiration. In all cases pyrophosphate raises the R.Q. With lactate as substrate pyrophosphate also causes a considerable increase in  $O_2$  uptake and a small increase in acid disappearance.

It will be shown in a forthcoming paper that the rate of succinate oxidation by ox retina is not great enough for it to be a link in the chain of reactions for the oxidation of lactate. That pyrophosphate does not inhibit but indeed accelerates lactate and acid removal is further evidence that this reaction does not go through succinate.

From these results one might assume that in liver, testis and kidney succinate is an intermediate in carbohydrate metabolism, and pyrophosphate, in inhibiting succinoxidase, reduces respiration. Perhaps in kidney there is an alternative path for glucose oxidation. However, in brain and retina the succinate-fumarate system does not seem to play a necessary role. In the case of these tissues inhibition of the succinoxidase system does not inhibit respiration or acid disappearance. Pyrophosphate seems to act catalytically in retina in the oxidation of glucose or lactate, perhaps by formation of cocarboxylase or other coenzyme or by formation of some phosphorylated intermediate.



## SUMMARY

Using the Dixon-Keilin manometric technique, it was found that pyrophosphate exerts various effects on the metabolism of tissue slices depending both on the tissue and the substrate employed.

1. It has no effect on the metabolism of brain slices.
2. The respiration of testis is inhibited in the presence or absence of added substrate.
3. Pyrophosphate has no effect on the normal respiration of tumour and, in low concentrations, has no effect on glucose oxidation, while in higher concentrations both glucose and lactate oxidations are inhibited.
4. It has little effect on the normal respiration of liver but inhibits respiration in the presence of glucose and lactate.
5. Pyrophosphate enhances the rate of glucose metabolism of kidney, has no effect on normal respiration and inhibits the complete oxidation of succinate, malate, pyruvate and lactate.
6. It has practically no effect on the normal respiration of retina but it considerably increases the rate of oxidation of glucose and lactate.

The authors wish to express their appreciation to Dr Ellice McDonald, Director, for his support and interest throughout the work, and to Dr K. A. C. Elliott for much helpful advice and criticism.

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## XVII. AN ULTRAMICRO-KJELDAHL TECHNIQUE

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*(Received 26 November 1938)*

THE problem of making Kjeldahl determinations of total nitrogen in amounts of the order of  $1\text{ }\mu\text{g.}$  is not an easy one, and has not yet been satisfactorily solved. The present paper records a contribution to this end which has proved extremely useful in work described elsewhere on the metabolism of isolated regions of the amphibian gastrula [Needham *et al.* 1939; Boell & Needham, 1939; Boell *et al.* 1939]. As an indication of the delicacy of technique required, it may be recalled that the Pregl methods need amounts of from 100 to  $300\text{ }\mu\text{g. N}$  [Pregl, 1937]. The amounts of total N which were to be estimated in the isolates from gastrulae varied from 1 to  $20\text{ }\mu\text{g.}$

Three previous papers have been devoted to this subject. Kirk [1934] worked out a method for Kjeldahl determinations on amounts of protein-N ranging from 3 to  $15\text{ }\mu\text{g.}$  His method involved a rather undesirable transfer from the incineration vessel to a complicated steam distillation apparatus. He titrated some  $300\text{ }\mu\text{l.}$  of distillate with  $0.05\text{ }N$  NaOH. Levy [1936] abandoned transference and steam distillation: after dilution and neutralization of the digest, he estimated the nitrogen colorimetrically with the aid of Nessler solution and a Pulfrich photometer. Colorimetric estimations permitted him to measure amounts down to  $0.4\text{ }\mu\text{g.}$ , but most investigators would prefer to use a titration method, if possible. Lastly, Bentley & Kirk [1936] also abandoned steam distillation and had recourse to the diffusion principle, originally perfected by Conway & Byrne [1933] for ammonia determinations. They too, however, transferred the distillate from incineration vessel to diffusion vessel.

The essence of the problem may be said to lie in combining in one technique the Kjeldahl digestion, which logically demands a long narrow tube, and the Conway diffusion, which logically demands a maximal liquid-air interface, and hence a shallow vessel. At the same time it is desirable to neutralize the digest in a closed system so that none of the  $\text{NH}_3$  first coming off shall escape.

The principle of our method lies in turning the Kjeldahl digestion vessel on its side after neutralization, and diffusing off the  $\text{NH}_3$  from the resulting relatively large liquid-air interface into a hanging film of acid in a tube fitted into the mouth of the Kjeldahl vessel.

The vessel used by us<sup>1</sup> is shown in Fig. 1(a). It consists of a digestion vessel with two bulbs blown in it (pyrex glass), some  $7.5\text{ cm.}$  long. At the top there is an opening ground to receive a small tube of about the same dimensions as those used by Linderström-Lang & Holter [1933] in their  $\text{NH}_3$  estimations. Like their tubes also, it is coated on the inside with high melting-point paraffin wax. We refer to it as the receptor tube.

The digestion of the tissue proceeds as follows. The source of nitrogen is placed or pipetted into the bottom of the Kjeldahl vessel and  $50\text{--}60\text{ }\mu\text{l.}$  of the digestion mixture added. The digestion mixture used by us was made up by

<sup>1</sup> Vessels of this kind can be obtained from Messrs C. Dixon, London, W.C.

adding 3 g. pure  $\text{CuSO}_4$ , 1 g. pure  $\text{K}_2\text{SO}_4$  and 0.1 g.  $\text{SeO}_2$  to 300 ml. pure conc.  $\text{H}_2\text{SO}_4$ . The tubes are then placed in an air oven at about  $120^\circ$  for *ca.* 3 hr. to drive off traces of water. At the end of that time a good deal of charring will be visible, and the digestion proper is carried out over pin flames constructed from old hypodermic needles (Fig. 1 (b)). Two glass beads are usually added, and we employ, as suggested by Levy, a mobile flame which the operator uses to heat the upper bulb when necessary. With the amounts of nitrogen used by us, digestion is complete in 3–5 min. If the tubes have been well heated in the air oven beforehand, and if they are constantly agitated during the heating by the use of the stem of the mobile flame, there is little danger of loss by sputtering, although as always where incinerations are concerned, care and practice cannot be dispensed with. When the liquid has become straw-coloured, the heating is continued for  $\frac{1}{2}$ –1 hr. over a pin flame about 2 mm. high.

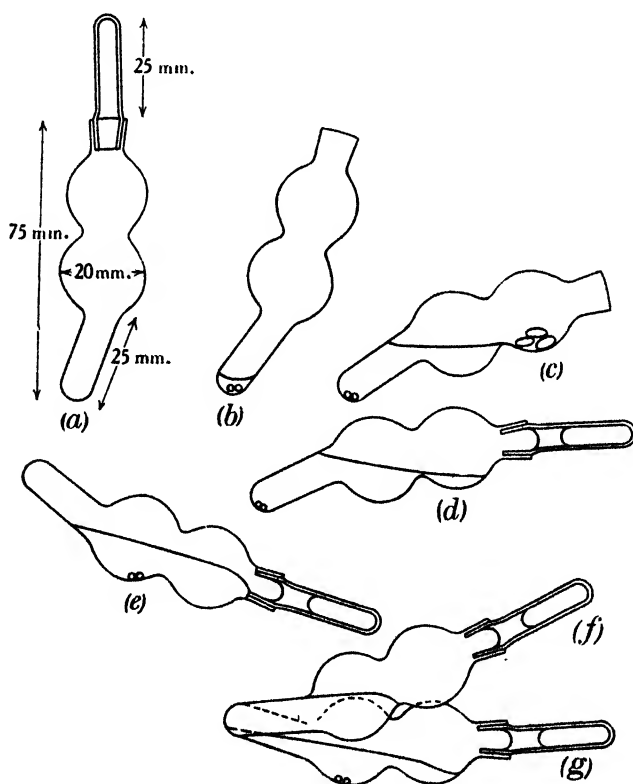


Fig. 1

The vessels are now removed from their holders and the digest diluted by the addition of 0.7 ml.  $\text{NH}_3$ -free distilled water. This is done with a pipette ending at an angle of  $45^\circ$  so as to facilitate the access of the water to all the interior surface of the vessel. The vessel is now tilted as shown in Fig. 1 (c) and three pellets of  $\text{NaOH}$  (corresponding to 300 mg. in all and giving 22 %  $\text{NaOH}$ ) are placed in the upper bulb.

Meanwhile the receptor tubes have been prepared. 70  $\mu\text{l.}$  of distilled water are placed as a hanging film at the top of the waxed interior and 7  $\mu\text{l.}$  of 0.294 *N*  $\text{HCl}$

delivered into the film from a constriction pipette [*vide* Linderstrøm-Lang & Holter, 1931]. The acid contains bromocresol purple in double the strength used in the Linderstrøm-Lang  $\text{NH}_3$  method. It is important to put enough water in the receptor tube, as owing to the osmotic pressure difference, the film loses water as it gains in ammonia, eventually falling to perhaps  $30\text{--}40\mu\text{l}$ . The receptor tube is well sealed into the Kjeldahl vessel with a grease composed of one part high melting-point paraffin wax and two parts white vaseline. The NaOH and the diluted digest are carefully mixed, care being taken to see that the heat evolved does not blow out the receptor tube, see Fig. 1(d). The whole is then cooled in water.

Now comes the most novel and delicate part of the technique. If traces of strong acid are left between the alkaline digest and the film of acid in the receptor tube, gross errors will result, for the ammonia will never reach its proper destination. The vessel is therefore held as in Fig. 1(e) and rotated so that the alkaline digest runs right round the opening of the receptor tube. The wax coating of the interior of the receptor tube, and the rim of wax grease around the ground joint alike prevent the entry of the slightest trace of strongly alkaline digest into the receptor tube. It is essential to cool the vessel well before carrying out this part of the technique, for otherwise the wax grease and the wax coating may melt, with unfortunate results.

It is now simply a question of time before the acid in the receptor tube is ready for titration. We place the vessels, however, in a horizontal position in a rocker at  $37^\circ$  overnight and titrate the following day. The rocker was easily made from an electric gramophone motor connected to an eccentric so as to give the motion seen in Fig. 1(f, g). At each slow rise and fall the alkaline digest spills into the upper bulb and then falls back again into the bottom of the vessel.

At the end of the period of diffusion desired, the receptor tubes are removed, the ends carefully wiped, and the films titrated against  $0.102\text{ N}$  NaOH from a Rehberg-Heatley micro-burette of  $50\mu\text{l}$ . capacity [*v.* Heatley, 1939]. Agitation of the film is accomplished magnetically as described by Linderstrøm-Lang & Holter [1931].

Using a solution of  $\text{NH}_4\text{Cl}$  we first determined the time taken under our conditions for 100 % of the  $\text{NH}_3$  to diffuse at  $37^\circ$  from the alkaline digest into the acid film in the receptor tube. As is shown by Fig. 2, 95.2 % recovery was reached

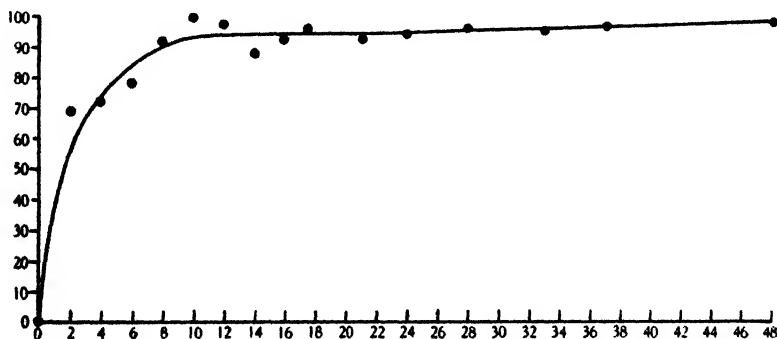


Fig. 2. Average recovery after 10 hours' distillation = 95.2 %.

in 10 hr. so that our usual practice of letting the rocking proceed for 18 hr. appears to be well on the safe side. The fact that the time required is a good deal longer than that in the  $\text{NH}_3$  methods of Conway & Byrne [1933] and Conway

[1935] must certainly be due to the fact that the Conway vessel has a large donator surface and a large receptor surface; under our conditions only a large donator surface is possible.

Table I shows the percentage recoveries from solutions of casein (Hammarsten).

Table I

Exp.	Casein ( $\mu\text{g.}$ )	Recovery ( $\mu\text{g.}$ )	Percentage
II	0.58	0.525	90
	1.17	1.11	95
III	1.13	1.19	105
	1.13	1.07	95
	1.14	1.12	98
I	2.06	2.36	115
	3.09	3.09	100
	3.09	3.11	101
	4.12	4.11	100
	6.57	5.83	89
	6.57	6.00	91
	6.57	6.56	100
	6.57	5.84	89
	6.57	5.85	89
	6.57	6.52	99
	6.57	6.25	95
	6.57	6.38	97
	6.57	6.62	101
	6.57	6.40	97
Av.			97.5

We see no reason why this method should not be applied to the estimation of quantities of total N much less than  $1\mu\text{g.}$ , but we believe that many further precautions would be necessary, especially as regards the purity of the reagents and the contamination of the atmosphere, as by smoking etc.

#### SUMMARY

An ultramicro-Kjeldahl technique, suitable for amounts of total N from 1 to  $20\mu\text{g.}$  is described. It includes a new type of Kjeldahl vessel, which, when turned on its side, acts as a Conway dish with a relatively large donator surface. The  $\text{NH}_3$  diffuses into an acid film hanging in a modified Linderström-Lang wax-coated receptor tube, which blocks up the mouth of the Kjeldahl vessel. Tests of the method, with a working description, are given in the text.

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## XVIII. THE VITAMIN C REQUIREMENTS OF MAN

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THE subject of this communication is a continuation of an inquiry into the relation of the intake of ascorbic acid to the urinary output and to the storage of the vitamin in the body.

The first paper of this series [Johnson & Zilva, 1934] dealt with the excretion of ascorbic acid by the kidney in man. The second inquiry [Zilva, 1936] was concerned not only with the excretion but with the storage of vitamin C in the body of the guinea-pig. In this animal only could storage questions be satisfactorily studied.

These investigations revealed that there was no standard of normality as regards the urinary excretion of ascorbic acid by man but that the output was conditioned by the amount stored in the body and by the quantity consumed in the diet. This conclusion was in agreement with the observations made by Van Eekelen *et al.* [1933] and by Hess & Benjamin [1934]. It was further found that when the store of the vitamin in the body was low, some time elapsed before a constant level of excretion indicating "saturation" was reached when the dose was small and that this time could be markedly shortened when large doses were offered. Quantities of ascorbic acid apparently larger than those required by the organism were not accounted for in the urine when the subject was "saturated" and the amount of vitamin thus not utilized was greater when larger doses were taken. On discontinuing the intake of ascorbic acid "saturation" persisted for a short time although the output in the urine almost disappeared. On the other hand, subjects only partially "saturated" could excrete considerable quantities of ascorbic acid when large doses were taken. The experiments on guinea-pigs disclosed in addition the fact that ascorbic acid first appeared in the urine when the maximum concentration of the vitamin was reached in the tissues. No doubt this also holds in the case of man.

These data brought into relief two interesting points. First, in order to determine the daily requirements of vitamin C by balancing the intake and the urinary excretion of ascorbic acid, the suitable dose should be established by trial and error. The dose should also be small, in order to reduce to a minimum the amount of ascorbic acid which, although not accounted for in the urine, is not actually involved in the metabolic function of the vitamin. Secondly, the minimum protective dose of ascorbic acid cannot be easily defined. It has been established that it is possible to exist for months on diets containing quantities of vitamin C sufficient to maintain good health and to prevent scurvy in spite of the fact that the store of ascorbic acid is very low. In guinea-pigs the daily dose required to bring about "saturation" is about 20 times that necessary to protect them from macroscopic scurvy. As a disparity between the two respective doses undoubtedly exists also in the case of man it becomes a debatable point as to what constitutes the optimum dose.

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The obvious extension of the work lay in the direction of ascertaining the minimum daily dose required to maintain a man "saturated" in respect to vitamin C, although, as will be pointed out later, such a dose is in all probability well above the optimum dose. With this end in view a number of experiments on the metabolism of vitamin C were carried out by the writers. In addition the ascorbic acid content of the blood was determined at various stages of these experiments in order to throw further light on the problem. The results obtained in the course of this investigation form the subject of this communication.

### *Methods*

As was already mentioned, an individual may be considered as "saturated" when the amount of ascorbic acid voided in the urine after a continued consumption of a certain dose becomes more or less constant. The first appearance of the acid is not always indicative of "saturation". The level of ascorbic acid excretion reached at "saturation" for a certain dose will, however, vary in certain instances according to the previous intake of ascorbic acid. If this has been high the level of excretion at equilibrium will be higher, at least for a very considerable time, than it would be if the intake had been lower. If on reaching "saturation" the intake of ascorbic acid is stopped, excretion persists for a few days whilst the output gradually diminishes. Therefore, on descending from a higher to a lower dose there is a certain amount of overlapping in the urinary output of the vitamin until, after a gradual diminution, a more or less constant level of excretion is attained. On the other hand, on ascending from a lower to a higher dose the output increases until equilibrium is reached. In order to obtain a more accurate conception, the level of excretion for each dose was determined by employing the dose both by ascending from a condition of "unsaturation" and by descending from a condition of "saturation" with as large a dose as 600 mg. per day. The doses employed were 30, 50 and 100 mg. since it was anticipated that the minimum daily dose capable of bringing about "saturation" lay within this range. The preliminary part of this investigation was carried out on S. S. Z. but for reasons of health it was decided to carry out the final experiments on A. E. K. (weight 184 lb.).

The indophenol titration procedure was similar to that employed by Johnson & Zilva [1934] who have previously shown by biological tests that under normal conditions the indophenol-reducing capacity could be ascribed mainly to ascorbic acid. There is, however, indirect evidence that not all of the reduction in the urine is necessarily due to ascorbic acid. Thus, in perusing the literature, one finds records of cases in which the urine from scorbutic patients reduced indophenol to some extent and this was assumed by some of the workers to be due to ascorbic acid. This assumption seems to be highly improbable, especially as it was shown by Johnson [1936] and by Kellie & Zilva [1938] that a reducing substance (or substances) which is not ascorbic acid persists in the lens of the eye and in the leucocytes of the guinea-pig after the remaining tissues of the animals have been exhausted of vitamin C by dietetic means. In the present investigation it was also found that when S. S. Z. and A. E. K. were "unsaturated" their urines reduced the indicator with comparatively little variation to the extent of 1 ml. N/1000 indophenol to 10 ml. of urine. This reduction value was assumed by us not to be due to ascorbic acid and was accordingly deducted from all the results.

The determination of ascorbic acid in the blood was carried out as follows. 10 ml. of blood were taken from a vein and collected directly into a centrifuge

tube containing 3 drops of saturated potassium citrate solution. The plasma which was obtained by centrifuging was then extracted with an equal volume of 10 % trichloroacetic acid and centrifuged. The extract was adjusted to pH 2-3 and titrated directly with 1.14 N/1000 indophenol. In one or two samples a certain amount of haemolysis took place during the collection of the blood. In these cases the trichloroacetic acid extract was adjusted to pH 4.5 and reduced with  $\text{H}_2\text{S}$  before being titrated. We should like to take this opportunity of thanking Dr C. R. Amies for collecting our samples of blood.

### Results

*Exp. 1.* In this experiment A. E. K., existing on a diet low in vitamin C, took a daily dose of 600 mg. of ascorbic acid until equilibrium was established in the urinary output. The dose was then reduced to 100 mg. It will be seen from Table I that during the first 3 days (10-12 September) on a daily dose of 100 mg. there was an overlapping in the output due to the extra excretion of the excess of ascorbic acid accumulated during the time when the subject was brought up to "saturation" by the daily consumption of 600 mg. A new level of excretion, namely 50-60 mg. per day, was then reached which persisted for 23 days. During part of this time, namely between 17 September and 4 October, the output was not measured, although the daily dose of 100 mg. was taken as usual. The measurement of the urinary excretion was resumed on 4 and 5 October, when it was found that the amount of ascorbic acid was still of the same order as that observed previously, indicating the probability that this level had been maintained all the time.

*Exp. 2.* In this experiment, which followed Exp. 1, the daily dose of ascorbic acid was reduced to 50 mg. and the output was studied for 17 days. The data (Table II) show that in this case the urinary excretion of ascorbic acid diminished very gradually to about 30 % of the intake, when it reached equilibrium after a fortnight. It will be seen later that a similar figure was obtained when this experiment was repeated a few months afterwards (Exp. 8).

Table I. *Ascorbic acid excretion in urine. Daily dose of 100 mg. following "saturation" on 600 mg. per day*

Date (1937)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
1 Sept.	600	1985	23.9	4.0
2	600	1678	249.5	41.5
3	600	1474	374.2	62.5
4	600		Not determined	
5	600		Not determined	
6	600	1067	462.6	76.8
7	600	1513	502.9	83.8
8	600	1293	526.0	87.6
9	600	1691	501.3	83.4
10	100	1942	110.6	110.6
11	100	1177	66.5	66.5
12	100	1915	75.1	75.1
13	100	1963	30.7	30.7
14	100	1450	48.8	48.8
15	100	1601	68.5	68.5
16	100	2137	44.3	44.3
17	100	1327	58.1	58.1
4 Oct.	100	1496	56.0	56.0
5	100	1401	58.4	58.4



Table II. *Ascorbic acid excretion in urine. Daily dose of 50 mg. following "saturation" on 100 mg. per day*

Date (1937)	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
6 Oct.	1309	76.3	152.6
7	1343	55.1	110.2
8	1095	36.0	72.0
9		Not determined	
10		Not determined	
11	1470	32.9	65.8
12	1147	31.1	62.2
13	1784	30.6	61.2
14	1041	26.9	53.8
15	1380	25.3	50.6
16		Not determined	
17		Not determined	
18	1529	26.4	52.8
19	1415	21.9	43.8
20	1435	16.7	33.4
21	1331	15.0	30.0
22	1547	15.1	30.2

Table III. *Ascorbic acid excretion in urine. Daily dose of 50 mg. following a period of "unsaturation"*

Date (1937)	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
29 Nov.	1415	2.5	5.0
30	1330	1.6	3.2
1 Dec.	1580	2.4	4.8
2	1410	2.5	5.0
3	1410	2.4	4.8
4		Not determined	
5		Not determined	
6	1844	2.1	4.2
7	1434	5.5	11.0
8	1555	4.2	8.4
9	1745	4.2	8.4
10	2029	5.1	10.2
11	2014	5.5	11.0
12		Not determined	
13	1601	5.2	10.4
14	1582	4.2	8.4
15	1910	3.4	6.8

Table IV. *Ascorbic acid excretion in urine. Daily dose of 30 mg. following "saturation" on 50 mg. per day*

Date (1937)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
16 Dec.	30	1604	2.2	7.3
17	30	1850	1.6	5.3
18	30		Not determined	
19	30		Not determined	
20	30	1678	1.1	3.7
21	30	1740	0.3	1.0
22	30	1501	0.4	1.3
23	600	1349	145.7	24.0
24	600	2430	331.4	55.0
25	600	1570	374.9	62.5

*Exp. 3.* The subject then placed himself on a diet deficient in vitamin C from 23 October to 28 November in order to attain a condition of "unsaturation". On 29 November (Table IV) he resumed his daily dose of 50 mg. of ascorbic acid. During the first 8 days only about 5 % of the intake was excreted by the kidney, but after that time the output in the urine rose to about 10 % and was maintained at this level for 9 days. The daily quantity of ascorbic acid thus excreted—as will also be seen from *Exp. 5*—was considerably less than that observed in *Exps. 2* and *8* when the same dose of the vitamin was taken after the subject had been previously "saturated" on higher doses, namely 600 mg. followed by 100 mg.

*Exp. 4.* This experiment, in which the daily dose of ascorbic acid was reduced to 30 mg., followed immediately on *Exp. 3*. After a week on this dose it was found (Table IV) that the output of the vitamin in the urine fell almost to zero. The subject at this time would appear to have been not far removed from "saturation", since of the 3 doses of 600 mg. taken on consecutive days 24 % was excreted on the first day, 55 % on the second and 62.5 % on the third.

*Exp. 5.* This experiment (Table V) was a repetition of *Exp. 3* in so far that the subject was not "saturated". Whilst, however, in *Exp. 3* he existed on a diet deficient in vitamin C for over a month, in this case his condition was not so far removed from "saturation", since he deprived himself of vitamin C for 8 days only after having been previously fully "saturated" (cf. *Exp. 4*). The daily output of ascorbic acid in the urine was, nevertheless, of the same order as in *Exp. 3*. The excretion of 82.2 % of a dose of 600 mg. of ascorbic acid after three daily consecutive doses afforded ample proof that the subject was "saturated".

*Exp. 6.* *Exp. 2* on the one hand, and *Exps. 3* and *5* on the other (daily dose 50 mg.), have shown that the level in the urinary excretion of ascorbic acid at equilibrium was dependent upon whether the patient was "saturated" or not at the beginning of the experiment. In *Exp. 6* an attempt was made to ascertain whether this held with a larger dose, namely 100 mg. The subject began taking this dose after having subsisted on a vitamin C-deficient diet for 6 days. The results in Table VI show that also on a dose of 100 mg., when the patient was only partially "saturated", the output of ascorbic acid in the urine at equilibrium was less than that when he was fully "saturated" (cf. Tables I and VII).

Table V. *Ascorbic acid excretion in urine. Daily dose of 50 mg. following a period of "unsaturation"*

Date (1938)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
4 Jan.	50		Not determined	
5	50	1308	3.4	6.8
6	50	1379	4.4	8.8
7	50	1217	4.2	8.4
8	50		Not determined	
9	50		Not determined	
10	50	1490	3.3	6.6
11	50	1784	2.7	5.4
12	50	1652	4.4	8.8
13	50	1602	4.6	9.2
14	50	1713	5.5	11.0
15	50		Not determined	
16	50		Not determined	
17	50	2041	3.3	6.6
18	600	1964	140.0	23.3
19	600	1683	374.0	62.3
20	600	1752	494.9	82.2

Table VI. *Ascorbic acid excretion in urine. Daily dose of 100 mg. following a period of "unsaturation"*

Date (1938)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
27 Jan.	100	Not measured	0	0
28	100	Not measured	0	0
29	100		Not determined	
30	100		Not determined	
31	100	1605	21.4	21.4
1 Feb.	100	1825	34.8	34.8
2	100		Not determined	
3	100	2180	39.2	39.2
4	100	1790	43.7	43.7
5	100		Not determined	
6	100		Not determined	
7	100	1805	48.3	48.3
8	100	2065	52.8	52.8
9	100	1814	38.9	38.9
10	100	2135	53.6	53.6
11	100	2107	44.2	44.2
12	100		Not determined	
13	100		Not determined	
14	100	2012	35.2	35.2
15	100	1975	49.7	49.7
16	100	1650	30.1	30.1
17	100	1950	30.0	30.0
18	100	1984	24.6	24.6
19	100	2100	39.3	39.3
20	100		Not determined	
21	100	1706	41.1	41.1
22	100	1802	39.2	39.2
23	100	2585	23.7	23.7
24	600	2229	427.4	71.2
25	600	1810	448.7	74.6
26	600	1760	543.7	90.6

Table VII. *Ascorbic acid excretion in urine. Daily dose of 100 mg. following "saturation" on 600 mg. per day*

Date (1938)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
27 Feb.	100	2090	137.8	137.8
28	100	1602	71.7	71.7
1 Mar.	100	1935	68.8	68.8
2	100	1735	72.4	72.4
3	100	1879	53.4	53.4
4	100	1669	49.4	49.4
5	100		Not determined	
6	100		Not determined	
7	100	1595	40.7	40.7
8	100	2415	50.8	50.8
9	100	2420	60.2	60.2
10	100	2017	43.4	43.4
11	100	1715	55.7	55.7
12	100		Not determined	
13	100		Not determined	
14	100	1180	37.8	37.8
15	100	1325	40.2	40.2
16	600	2442	409.1	68.0
17	600	1228	462.8	77.0
18	600	1620	481.2	80.1

Table VIII. *Ascorbic acid excretion in urine. Daily dose of 50 mg. following "saturation" on 600 mg. per day*

Date (1938)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
19 Mar.	50		Not determined	
20	50		Not determined	
21	50	1300	31.1	62.2
22	50	2125	17.7	35.4
23	50	2005	18.6	37.2
24	50	1825	13.8	27.6
25	50	2195	10.7	21.4
26	50		Not determined	
27	50		Not determined	
28	50	1734	9.9	19.8
29	50	1845	13.0	26.0
30	50	2164	13.9	27.8
31	50	1945	10.1	20.2
1 Apr.	50	1987	11.3	22.6
2	50		Not determined	
3	50		Not determined	
4	50	1922	13.2	26.4
5	600	2015	224.1	37.3
6	600	1750	339.5	56.5
7	600	2287	513.8	85.4

*Exp. 7.* This experiment was a repetition of Exp. 1 and was carried out immediately after Exp. 6. The subject was then fully "saturated" (cf. Table VI). The urinary excretion at equilibrium was found to approximate (Table VII) to that observed in Exp. 1 (Table I) and not to that found in Exp. 6 when the subject was not "saturated".

*Exp. 8.* In this experiment, performed immediately after Exp. 7, the dose was reduced to 50 mg. It was therefore a repetition of Exp. 2. Although equilibrium in the urinary excretion of ascorbic acid was attained in this case (Table VIII) earlier than in Exp. 2, the output at equilibrium was of the same order in both experiments.

Tables IX-XI give the ascorbic acid contents of the blood of the two experimental subjects during the course of the investigation. These figures yield information particularly concerning two points. Without going into a detailed analysis of the literature on the vitamin C content of the blood, which is very extensive and often contradictory, it may be stated that some workers maintain that there is a renal threshold for ascorbic acid in man and that the ascorbic acid concentration of the blood is an index of the degree of "saturation" of the subject in respect to vitamin C. The data in Tables IX-XI throw some light on both these problems.

The fact that a subject is capable of excreting ascorbic acid in the urine before reaching "saturation" when the dose taken is large, suggests that the concentration of the vitamin in the blood must to some extent control its excretion by the kidney. The threshold values advanced by a number of workers vary from 0.9 mg. to 1.5 mg. per 100 ml. of blood. Even if the individual variation of the subject and the variation in the methods employed by these workers be taken into account, the difference in the results obtained by them would still seem to be too great. Our results may offer some explanation for this disparity. It is seen that when S. S. Z. (23 February 1937, Table IX), on reaching "saturation", discontinued his dose, the ascorbic acid content of his blood then fell from 1.63 mg. per 100 ml. of plasma to 0.45 mg. when the dose was 600 mg.

Table IX. *Relation of the ascorbic acid content of the blood of "saturated" subjects to their 24 hr. urinary output*

Subject and date (1937)	Time of collection	Ascorbic acid in plasma mg./100 ml.	Ascorbic acid in urine mg./24 hr.	Remarks
S. S. Z.				
23 Feb.	11.25 a.m.	1.63	525.1	"Unsaturated" level of ascorbic acid =0.35 mg./100 ml. plasma. 15-23 Feb. (incl.) 600 mg. ascorbic acid daily at 10 a.m.
24	11.35 a.m.	0.80	74.9	
25	12 noon	0.53	40.9	
26		Not determined	18.6	
27	11.35 a.m.	0.45	Not determined	
			mg. excreted from 10 a.m. to 5 p.m.	
8 Mar.	12.30 p.m.	0.73	16.6	27 Feb.-8 Mar. (incl.) 100 mg. ascorbic acid daily at 10 a.m.
9		Not determined	21.9	
10	11.15 a.m.	0.54	7.2	
A. E. K.				
9 Sept.	11 a.m.	1.53	501.3	"Unsaturated" level of ascorbic acid -0.47 mg./100 ml. plasma. 1-9 Sept. (incl.) 600 mg. ascorbic acid daily at 11 a.m.
17	10 a.m.	1.28	58.1	
15 Oct.	10 a.m.	0.80	25.3	10-17 Sept. (incl.) 100 mg. ascorbic acid daily at 10 a.m.
21	10 a.m.	1.08*	15.0	18 Sept.-3 Oct. (incl.) 2 oranges daily.
22	10 a.m.	1.06	15.1	4-5 Oct. (incl.) 100 mg. ascorbic acid daily. 6-15 Oct. (incl.) 50 mg. ascorbic acid daily at 10 a.m.
14 Dec.	10 a.m.	0.47*	4.2	16-22 Oct. (incl.) 50 mg. ascorbic acid daily at 10 a.m.
15	10 a.m.	0.62	3.4	23 Oct.-28 Nov. (incl.) vitamin C-deficient diet. 29 Nov.-15 Dec. (incl.) 50 mg. ascorbic acid daily at 10 a.m.
22	10 a.m.	0.67	0.4	16-22 Dec. (incl.) 30 mg. ascorbic acid daily at 10 a.m.

\* Some haemolysis.

and from 0.73 to 0.54 mg. when the dose was 100 mg., i.e. to values considered by many workers as an indication of vitamin C deficiency. In spite of these low blood levels eventually reached, the subject was fully "saturated" and still excreted ascorbic acid in the urine. It would therefore appear that in this case the level of the so-called "renal threshold" was influenced by the ascorbic acid content of the tissues. A similar conclusion can be drawn from A. E. K.'s data (Table IX). That the excretion of ascorbic acid by the kidney is not entirely influenced by the blood ascorbic acid level is apparent when a comparison is made between the blood content and the quantities of ascorbic acid excreted by "saturated" and "unsaturated" subjects immediately before and after the samples of the blood were taken (Tables X and XI). These results suggest that in all probability there is a competition for the ascorbic acid of the blood by the absorptive tendency of the tissues and the excreting function of the kidney.

The results also show that blood levels determined at random, even if they be "fasting" values, may sometimes lead to wrong conclusions concerning the condition of "saturation" of the subject. The data given in Tables IX–XI

Table X. *Relation of the ascorbic acid content of the blood of "saturated" subjects to their urinary output*

Subject and date (1937)	Ascorbic acid intake		Time of collection	Ascorbic acid		Remarks
	Time	Dose mg.		Plasma mg./100 ml.	Urine mg. sample	
S. S. Z.						
23 Feb.	10 a.m.	600	11.25 a.m. 12.45 p.m. 2.30 p.m.	1.63 Not determined 1.45	25.9 65.3 121.6	"Unsaturated" level, 0.35 mg./100 ml. plasma. 15-22 Feb. (incl.) 600 mg. ascorbic acid daily. Subsequent urinary excretion: 2.30-5 p.m. 113.7 mg.; 5-10 p.m. 103.1 mg.; 10 p.m.-8 a.m. (24 Feb.) 73.8 mg.; 8-10 a.m. 21.7 mg.
A. E. K.						
9 Sept.	11 a.m.	600	11 a.m. 12 noon 2 p.m.	1.51 Not determined 2.89	No urine passed 6.1 189.5	"Unsaturated" level, 0.47 mg./100 ml. plasma. 1-8 Sept. (incl.) 600 mg. ascorbic acid daily. Subsequent urinary excretion: 2-4.30 p.m. 174.8 mg.; 4.30-6 p.m. 54.9 mg.; 6-9 p.m. 40.2 mg.; 9-11 p.m. 15.4 mg.; 11 p.m.-12.20 a.m. (10 Sept.) 6.4 mg.; 12.20-10 a.m. 14 mg.
17 Sept.	10 a.m.	100	10 a.m. 12 noon 1 p.m.	1.28 Not determined 1.71	0.2 2.1 No urine passed	10-16 Sept. (incl.) 100 mg. ascorbic acid daily. Subsequent urinary excretion: 12-2 p.m. 4.5 mg.; 2-4 p.m. 18.3 mg.; 4-6.30 p.m. 17.2 mg.; 6.30-9 p.m. 6.3 mg.; 9 p.m.-8 a.m. (18 Sept.) 8.2 mg.; 8-10 a.m. 1.5 mg.
22 Oct.	10 a.m.	50	10 a.m. 12 noon 1 p.m.	1.06 Not determined 1.29	0.7 1.0 No urine passed	18 Sept.-3 Oct. (incl.) 2 oranges daily; 4-5 Oct. (incl.) 100 mg. ascorbic acid daily; 6-21 Oct. (incl.) 50 mg. ascorbic acid daily. Subsequent urinary excretion: 12-2 p.m. 2.1 mg.; 2-4 p.m. 2.8 mg.; 4-6 p.m. 1.0 mg.; 6-10 p.m. 2.5 mg.; 10 p.m.-8 a.m. (23 Oct.) 5.2 mg.; 8-10 a.m. 0.5 mg.
15 Dec.	10 a.m.	50	10 a.m. 12 noon 1 p.m.	0.62 Not determined 0.80	0 0 No urine passed	23 Oct.-28 Nov. vitamin C-deficient diet. 29 Nov.-14 Dec. (incl.) 50 mg. ascorbic acid daily. Subsequent urinary excretion: 12-2 p.m. 1.2 mg.; 2-4 p.m. 0.4 mg.; 4-6 p.m. 0.3 mg.; 6-10 p.m. 0 mg.; 10 p.m.-8 a.m. (16 Dec.) 1.4 mg.; 8-10 a.m. 0.1 mg.
22 Dec.	10 a.m.	30	10 a.m. 12 noon 1 p.m.	0.67 Not determined 0.72	0 0 0	16-21 Dec. (incl.) 30 mg. ascorbic acid daily. Subsequent urinary excretion: 1-10 p.m. 0 mg.; 10 p.m.-8 a.m. (23 Dec.) 0.4 mg.; 8-10 a.m. 0 mg.

Table XI. Relation of the ascorbic acid content of the blood of an "unsaturated" subject (A. E. K.) to his urinary output

Date (1937)	Time of collection	Ascorbic acid		Date (1937)	Time of collection	Ascorbic acid	
		Plasma mg./100 ml.	Urine mg./sample			Plasma mg./100 ml.	Urine mg./sample
26 Jan.	10 a.m.	Not determined	0	26 Aug.	10 a.m.-6 p.m.	Not determined	0
	1 p.m.	"	0	27	10 a.m.-6 p.m.	"	0.3
	2.15 p.m.	0.57	No urine passed	28	10 a.m.-12 noon	"	0.3
	4.45 p.m.	Not determined	0	29	10 a.m.-6 p.m.	"	0.7
	6 p.m.	"	0	30	100 mg. ascorbic acid taken at 11 a.m.	"	"
27	600 mg. ascorbic acid taken at 10 a.m.						
	10.30 a.m.	0.92	0		10 a.m.	Not determined	0
	11 a.m.	Not determined	0		11 a.m.	0.52	No urine passed
	11.45 a.m.	"	0		12 noon	0.67	0
	12.25 p.m.	"	0		2 p.m.	0.72	0
	12.45 p.m.	1.27	No urine passed		4 p.m.	Not determined	0
	1.15 p.m.	Not determined	0		6 p.m.	"	0
	2.30 p.m.	"	0	31 Aug.	10 p.m.	"	0
	3.15 p.m.	"	0		8 a.m.	"	0
	4 p.m.	1.12	No urine passed		10 a.m.	"	0
28	7 p.m.	Not determined	2.1		11 a.m.	0.50	0
	10.30 p.m.	"	0		12 noon	Not determined	0
					2 p.m.	"	0
	8.30 a.m.	"	3.2		4 p.m.	"	0
	10 a.m.	1.0	No urine passed		6.15 p.m.	"	0
	10.45 a.m.	Not determined	1.2		10 p.m.	"	0
	1 p.m.	"	0	1 Sept.	8 a.m.	"	1.1
	3.20 p.m.	"	0.3		600 mg. ascorbic acid taken at 10.30 a.m.		
	5 p.m.	0.83	No urine passed		10.30 a.m.	0.47	0
	5.50 p.m.	Not determined	1.8		12 noon	Not determined	0.4
29	8.30 p.m.	"	0.4		1.30 p.m.	1.69	No urine passed
	10 a.m.	"	0.5		2 p.m.	Not determined	7.5
	12.30 p.m.	0.77	0		4 p.m.	"	11.6
					6 p.m.	"	2.9
					8 p.m.	"	0
				2 Sept.	10.30 p.m.	"	0.8
					2.45 a.m.	"	0
					10 a.m.	"	1.5
					10.30 a.m.	1.10	No urine passed

afford further information about the variation of the ascorbic acid content of the blood with the magnitude of the dose and the time elapsing after taking the dose. They show, as has already been observed by numerous workers, that the vitamin C content of the blood rises after oral administration of the vitamin, reaching a maximum 2-4 hr. later. The maximum value thus attained is influenced by the magnitude of the dose and by the "fasting" value; it also varies with the individual.

### *Conclusions*

The experiments bring out very plainly the difference between the level of excretion of ascorbic acid at equilibrium when a certain dose was taken after ascending from a condition of "unsaturation" or from a condition of "saturation" reached on a smaller dose and that recorded with the same dose taken after having previously attained "saturation" with a larger dose. This disparity in the level of output no doubt explains, at least in part, the variation in the amount of the apparent retention observed by different workers on the same dose of ascorbic acid.

As was to be expected, these levels of excretion decreased with the diminution in the intake. On a daily dose of 30 mg. the appearance of ascorbic acid in the urine almost ceased. On the other hand, on a dose of 50 mg. 20-30% of the intake was excreted by the kidney at equilibrium when descending from a higher dose and 8-10% when ascending from "unsaturation". From this it may be assumed that the minimum dose necessary to bring about "saturation" lies in this case between 30 and 50 mg. per day, probably nearer the former figure. Schultz [1937] was able to "saturate" a scorbutic patient (weight 63 kg.) in 4 weeks with a daily dose of 40 mg. of ascorbic acid administered intravenously. In all probability ascorbic acid is utilized more efficiently when introduced parenterally than when it is given orally.

Although in practice it is advisable to aim at a dose of this order it is by no means to be assumed that this is the minimum daily dose necessary to prevent the onset of scurvy or even to maintain good health. It would perhaps be appropriate at this stage to review a few facts established in this connexion concerning the vitamin C requirements of the guinea-pig. Zilva, by determining the ascorbic acid content of the tissues of these animals, found that the condition of "saturation", i.e. when the tissues carry the maximum load of the vitamin, could only be maintained by administering *per os* a daily dose of 20 mg. or more. When the dose was reduced to about 8 mg. the tissues lost after 15 days the major part of their ascorbic acid, only traces being present; yet this dose is 4 times the daily minimum dose necessary to protect a guinea-pig fully from macroscopic and microscopic scorbutic lesions. On the assumption that the same proportions hold in the case of man one would expect that about a half of the "saturation" dose, i.e. 15 mg., would be sufficient not only to prevent scurvy but to maintain an individual in good health. This dose may at first sight appear to be rather low. On reflexion, however, it will be found that in certain circumstances the daily food of apparently healthy individuals does not contain over long periods much more vitamin C than this. The above figure of 15 mg. cannot therefore be far removed from the actual theoretical requirement of man. The view advanced by one of us [Zilva, 1936; 1937] may here be stressed once more, that no undue clinical significance need be attached to the importance of maintaining the condition of "saturation". As already mentioned, however, in order to ensure a margin of safety, it may be advisable to insist in practice on the minimum "saturation" dose, which in this investigation was found to be



30–40 mg. per day, as the requisite vitamin C intake for healthy adults. In the case of pregnant women and of nursing mothers this would of course be considerably higher.

#### SUMMARY

The level of urinary excretion of ascorbic acid on a certain dose was found to be lower after ascending from a condition of "unsaturation", or from a condition of "saturation" reached on a lower dose, than when the same dose was taken after having previously attained "saturation" with a higher dose.

The equilibrium in the excretion of a subject weighing 184 lb. was established on the following daily doses, 30 mg., 50 mg. and 100 mg.

On a daily intake of 30 mg. the appearance of ascorbic acid in the urine almost ceased. It was assumed that the minimum "saturation" dose lay in the neighbourhood of this figure.

Although in practice it would be advisable to recommend the "saturation" dose as the minimum vitamin C daily intake for normal individuals it is argued that about half of this dose is sufficient not only to prevent scurvy but to maintain them in good health.

The ascorbic acid content of the blood was determined in two individuals during "saturation" and "unsaturation" at different intervals after the administration of the vitamin in various doses. The comparison of these figures with those of the urinary excretion suggests that there is no constant renal threshold for ascorbic acid, but that there is a competition for the ascorbic acid of the blood by the absorptive capacity of the tissues and the excreting function of the kidney.

Blood levels determined at random do not indicate the degree of "saturation" of a subject.

One of us (A. E. K.) is indebted to the Medical Research Council for a whole time grant. We should also like to express our thanks to Messrs Roche Products Ltd. for a gift of ascorbic acid.

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# XIX. THE ACTION OF SOME *ENDOSUCCINIC* ACIDS DERIVED FROM POLYCYCLIC HYDROCARBONS ON THE RED BLOOD CORPUSCLES OF THE MOUSE

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*(Received 17 December 1938)*

STRONG [1936] and Strong & Francis [1937] have described a fall in the haemoglobin content of the blood of female mice of strains which are liable to the development of spontaneous mammary carcinoma. The experiments of Parsons and her collaborators have shown that general irradiation of mice or rats with sub-lethal doses of X-rays increases and accelerates tumour production on subsequent treatment of the animals with a carcinogenic compound [Mayneord & Parsons, 1937; Clarkson *et al.* 1938; Parsons, 1938]. This change produced by the X-rays is one affecting the haemopoietic system; the red blood cells are diminished (in rats) and iron is deposited in the lymph glands.

Parsons and her fellow-workers have also shown that the same signs of blood destruction are present in mice undergoing treatment with a carcinogenic substance. Application of the prussian blue reaction to sections of the lymph glands of these animals before the appearance of tumours revealed the presence of iron. In a forthcoming publication the present author will adduce quantitative estimations of iron in support of these microscopical observations.

In view of these results it was considered of interest to carry out some simple *in vitro* experiments on the action of the compound used by Parsons and co-workers. The experiments were ultimately extended to embrace a series of similar compounds derived from carcinogenic and closely related non-carcinogenic hydrocarbons. The choice of the hydrocarbons to be used was necessarily restricted to those which yield adducts with maleic anhydride. For example, phenanthrene and 3:4-benzphenanthrene were excluded by this condition and, so far, all efforts to obtain an adduct from 3:4-benzpyrene have been unsuccessful.

## MATERIALS

The compound used by Parsons and co-workers was sodium 1:2:5:6-dibenzanthracene-9:10-*endo*- $\alpha\beta$ -succinate [Cook, 1931]. Diels & Alder [e.g. 1931] and Clar [1932] have shown that hydrocarbons of the anthracene type form adducts with maleic anhydride. More recently Bachmann & Kloetzel [1938] have shown that the reaction is a balanced one and have described a procedure for obtaining good yields of the adducts from several important carcinogenic hydrocarbons.

In the present work the solutions of the Na salts of the *endosuccinic* acids were prepared as follows. The anhydrides were prepared by boiling the hydrocarbon (1 part) in xylene or benzene (100 parts) with maleic anhydride (10 parts). After distillation of the solvent the anhydride was hydrolysed by warming for a few minutes with conc. KOH, the alkaline solution diluted with a large volume of water, warmed to effect solution of the K salt and filtered from unchanged

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hydrocarbon. On acidification of the filtrate the free acid was precipitated and collected. Solutions of the Na salts of the *endosuccinic* acids were prepared by warming a slight excess of the acid with a measured volume of *N*/10 NaOH and filtering from the excess acid. Sufficient NaCl was added to the filtrate to make the final concentration 0.85 % (the effect of the *endosuccinate* on the tonicity of the solution may be neglected since its concentration is low and its mol.wt. is high).

In a few cases this procedure had to be modified as the free acid on precipitation rapidly dehydrated to the anhydride which was insoluble in dilute NaOH. In these cases advantage was taken of the fact that the Na salts are very sparingly soluble in presence of NaCl and, after hydrolysing the anhydrides with conc. NaOH, the salt of the *endosuccinic* acid was precipitated by saturating the solution with NaCl. The Na salt was collected and well washed with sat. NaCl and finally with 0.85 % NaCl to remove excess alkali.

The solubilities of the Na salts in 0.85 % NaCl vary considerably. In the group consisting of the derivatives of 1:2:5:6-dibenzanthracene (*cis* compound), 1:2:3:4-dibenzanthracene, 5:6-*cyclopenteno*-1:2-benzanthracene, 9:10-dimethyl-1:2-benzanthracene, cholanthrene and methylcholanthrene, the solubility in cold saline is not very great and the stock solutions consisted of saturated solutions of their salts. Anthracene, naphthacene, 1:2-benzanthracene and the remaining methyl-substituted 1:2-benzanthracenes employed gave Na salts which were relatively easily soluble and stock solutions of these were prepared containing 2 mg. per ml. (0.2 %). The *trans*-acid from 1:2:5:6-dibenzanthracene also gave a readily soluble Na salt.

In all cases the solutions used for the haemolytic tests had a *pH* of approximately 7.2.

The compounds used in this investigation are given in the following list under the name of the parent hydrocarbon. With one exception all the *endosuccinic* acids used have the normal (*cis*) configuration. Reference is made below to their method of preparation and in the case of six members of the series which have not been hitherto described, analytical specimens of the anhydrides were isolated and the results of their elementary analyses are given. All melting points are uncorrected.

*Anthracene*. Prepared from anthracene by the method of Diels & Alder [1931]. The Na salt is easily soluble in saline.

*Naphthacene*. The procedure given by Clar [1932] was followed. The Na salt is readily soluble.

*1:2-Benzanthracene*. Prepared according to Clar's directions. Na salt comparatively easily soluble.

*1:2:5:6-Dibenzanthracene*. The normal (*cis*) compound was prepared according to Bachmann's improved method. Conversion into the stereoisomeric *trans*-compound was effected in the manner described by Diels & Alder [1931] for the corresponding anthracene adduct. The *cis*-acid was converted into the *cis*-dimethyl ester [Bachmann & Kloetzel, 1938] and the latter rearranged by boiling in alcoholic solution with Na. The *trans*-ester which is formed as an intermediate is hydrolysed by the excess of  $\text{NaOC}_2\text{H}_5$  to the *trans*-Na salt. The free *trans*-acid was liberated from the Na salt by acidification. A specimen of this acid on recrystallization from dilute acetic acid melted at 255–257° and gave a depression on mixing with a specimen of the pure *cis*-acid (m.p. 230°). The solubility of the Na salt of the *trans*-acid is very much greater than that of the *cis*-acid.

For further characterization a specimen of the *trans*-acid was converted into the dimethyl ester by means of diazomethane. The ester crystallized from

methyl alcohol in prisms, m.p. 179–180°. (The corresponding *cis*-ester has m.p. 230–231°). Found: C, 79.5; H, 5.6%.  $C_{28}H_{22}O_4$  requires: C, 79.6; H, 5.25%.

*1:2:3:4-Dibenzanthracene*. The adduct was prepared in xylene. The xylene solution was cooled in the ice-chest overnight and the solid which separated was collected and recrystallized twice from xylene. Clusters of thick needles were obtained, m.p. 250–251°. Found: C, 82.85; H, 4.3%.  $C_{26}H_{16}O_3$  requires: C, 83.0; H, 4.3%. The Na salt of the acid is sparingly soluble in saline.

*5:6-cyclo-Penteno-1:2-benzanthracene*. The free acid was prepared by the general procedure described above. On boiling a solution of the free acid in acetic anhydride for a short time and then cooling the solution the anhydride slowly crystallized in stout needles. After two recrystallizations from acetic anhydride the crystals melted at 245–246°. Found: C, 81.5; H, 5.0%.  $C_{25}H_{18}O_3$  requires: C, 81.7; H, 5.0%. The Na salt of this acid is sparingly soluble in saline.

*Cholanthrene*. The adduct was prepared in benzene. The solid which separated on cooling the benzene solution was recrystallized from ethyl acetate. The anhydride crystallized in rectangular plates melting at 219–220°. Found: C, 81.5; H, 4.7%.  $C_{24}H_{16}O_3$  requires: C, 81.8; H, 4.6%. The Na salt is sparingly soluble.

*Methylcholanthrene*. A specimen of the anhydride supplied by Dr W. E. Bachmann was converted into the Na salt of the acid. The salt is sparingly soluble.

*9:10-Dimethyl-1:2-benzanthracene*. A specimen of the anhydride was furnished by Dr Bachmann. The Na salt is sparingly soluble.

*3-Methyl-1:2-benzanthracene*. The free acid was prepared in the usual way. Treatment with acetic anhydride and recrystallization from the same solvent yielded the anhydride as prisms melting at 257–258°. Found: C, 80.9; H, 4.8%.  $C_{23}H_{16}O_3$  requires: C, 81.15; H, 4.7%. The Na salt is relatively easily soluble.

*5-Methyl-1:2-benzanthracene*. Prepared as for the 3-methyl compound. The anhydride crystallized from acetic anhydride in fine needles melting at 252–253°. Found: C, 81.1; H, 4.8%.  $C_{23}H_{16}O_3$  requires: C, 81.15; H, 4.7%. Na salt relatively easily soluble.

*10-Methyl-1:2-benzanthracene*. The anhydride prepared from the free acid by boiling with acetic anhydride crystallized from this solvent in prisms melting at 262–264°. Found: C, 81.0; H, 5.0%.  $C_{23}H_{16}O_3$  requires: C, 81.15; H, 4.7%. The Na salt is relatively easily soluble.

*Other methyl-substituted 1:2-benzanthracenes*. Solutions of the sodium salts of the *endosuccinic* acids of 4-, 6-, 2', and 3'-methyl-1:2-benzanthracenes and also of 2':7-dimethyl-1:2-benzanthracene were prepared without isolating the intermediate anhydrides. The Na salts of these acids are all relatively easily soluble in 0.85% NaCl.

#### TECHNIQUE OF THE HAEMOLYSIS EXPERIMENTS

Mice were killed by breaking the neck and the blood from the heart drawn off with a syringe as quickly as possible. The blood was discharged into a small volume of 0.85% NaCl, centrifuged, the red cells washed twice with saline and suspended in a small volume of 0.85% NaCl.

The aqueous solutions of the Na salts were set up in 4 small tubes; the first contained the "stock" concentration (2 mg. per ml. in the case of the more soluble salts and a saturated solution in the case of those salts which were not soluble to this extent), and the remaining 3 aliquot dilutions of the first. A fifth tube contained only 0.85% NaCl as a control. To each of the 5 tubes two drops

of the red blood cell suspension were added and the tubes inverted once or twice to ensure mixing. The tubes were stoppered and observed at intervals for 24 hr. at room temperature.

In the control tubes the upper limit of the sinking red cells was quite sharply defined, and later these formed a compact deposit. In the experimental tubes the first sign of haemolysis was often a diffuseness in the upper limit of the falling red cells and the fluid above had a yellow tinge. Where the compound was very actively haemolytic, lysis occurred before any appreciable settling of the red cells had taken place and the first observable change was the increasing transparency as compared with the opaque suspensions in the control tubes.

### RESULTS

Using the simple technique outlined above the haemolytic actions of the 18 compounds previously mentioned were tested. Broadly speaking the activities of the compounds fall into three groups. In the 1st are those compounds which haemolysed rapidly and gave clear red transparent solutions within a matter of minutes. The members of this group are all sparingly soluble salts so it was not possible to arrange them in any order of activity as the saturated solutions employed were not necessarily of equal concentrations. In the 2nd group are those compounds which showed a lower degree of activity, and an approximate estimate of the relative potencies of these compounds could be obtained since the stock solutions of all of them contained 2 mg. per ml. In the 3rd group are three compounds which showed no evidence of haemolytic action even though left in contact with the red cells for 24 hr.

Table I gives a summary of the results for these three groups.

Table I	
	Haemolytic activity
Group I. (Saturated solutions)	
<i>endo</i> Succinates from:	
1:2:5:6-Dibenzanthracene ( <i>cis</i> and <i>trans</i> )	Strongly haemolytic
1:2:3:4-Dibenzanthracene	" "
5:6- <i>cyclo</i> -Penteno-1:2-benzanthracene	" "
Cholanthrene	" "
Methylcholanthrene	" "
9:10-Dimethyl-1:2-benzanthracene	" "
Group II. (Solutions 0.2% and less)	
<i>endo</i> Succinates from:	
2':7-Dimethyl-1:2-benzanthracene	+ + + + +
5-Methyl-1:2-benzanthracene	+ + +
6-Methyl-1:2-benzanthracene	+ + +
10-Methyl-1:2-benzanthracene	+ + +
3'-Methyl-1:2-benzanthracene	+ + +
3-Methyl-1:2-benzanthracene	+ +
2'-Methyl-1:2-benzanthracene	+ +
4-Methyl-1:2-benzanthracene	+
Group III. (Solutions 0.2% and less)	
<i>endo</i> Succinates from:	
Anthracene	Inactive
Naphthacene	"
1:2-Benzanthracene	"

### DISCUSSION

The first point of interest in considering the results is any possible correlation between the haemolytic activities of the *endosuccinic* acids and the known carcinogenic activities of the parent hydrocarbons. (It should be noted that the

water-soluble derivatives themselves have been tested for cancer-producing action in only three cases—the derivatives from 1:2:5:6-dibenzanthracene (*cis* and *trans*) and methylcholanthrene; all these produced sarcomas in mice.)

In Group I of the Table, 5 of the 6 hydrocarbons are potent carcinogens, the exception being 1:2:3:4-dibenzanthracene which in this laboratory produced no tumours when applied to the skin of a series of 20 mice, of which only 2 lived for more than a year, the last mouse dying on the 487th day. In an earlier experiment 3 epitheliomas were obtained in 3 out of 70 mice with an impure specimen of the hydrocarbon, the earliest tumour appearing after 619 days. No reports on this compound from other laboratories have been found in the literature. In Group II, the 2':7-dimethyl-, 2'-methyl- and 3'-methyl-1:2-benzanthracenes have not yielded tumours in any experiments recorded as yet. The 5 remaining hydrocarbons in this group are definitely carcinogenic, but, with the exception of the 10-methyl derivative, inferior in carcinogenic potency to the active hydrocarbons of Group I. In Group III anthracene and naphthacene have not given tumours in any experiments as yet reported, while 1:2-benzanthracene produced 1 epithelioma when applied to the skin of 30 mice, although negative results have been obtained in another series of 50 mice.

In general it may be said, for the 18 compounds tested, that the water-soluble salt derived from a hydrocarbon which is known to be carcinogenic is haemolytic but that the converse is not necessarily true and hydrocarbons which have not been shown to be carcinogenic may give rise to haemolytic *endo-succinic* acids.

#### SUMMARY

The Na salts of the *endosuccinic* acids derived from 17 polycyclic hydrocarbons have been examined for haemolytic action on mouse red blood cells. Haemolytic action was shown by the water-soluble derivatives from all hydrocarbons with a pronounced carcinogenic activity. Haemolysis was also shown by the derivatives of some hydrocarbons which have not been found to be carcinogenic.

I am indebted to Dr L. D. Parsons, who suggested this investigation, for much help with the haemolysis experiments and to Dr W. E. Bachmann and to Prof. J. W. Cook for the gift of compounds. It is a pleasure to express my thanks to the Sir Halley Stewart Trust for a Fellowship held during this work and to the British Empire Cancer Campaign for generous grants which have supported this investigation.

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## XX. *l*-(+)**CITRULLINE**

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**CITRULLINE** ( $\alpha$ -amino- $\delta$ -carbamido-*n*-valeric acid) was first encountered as a constituent of the juice of the water-melon [Koga & Odaki, 1914; Wada, 1930]. Wada not only elucidated its constitution but produced it synthetically from ornithine. The substance was subsequently obtained by the action of putrefactive bacteria [Ackermann, 1931] and of *Bacillus pyocyaneus* [Horn, 1933] upon arginine, and also as a product of the tryptic digestion of casein [Wada, 1933]. More recently, methods have been described of preparing it in larger quantity from *dl*-ornithine [Kurtz, 1938] or from arginine [Fox, 1938].

Ackermann gives no information concerning the behaviour of his preparation towards polarized light, nor does Wada [1933] in the case of the product obtained from casein. All other specimens of citrulline hitherto prepared are described as optically inactive. The present paper describes for the first time the preparation of dextrorotatory citrulline and the determination of its specific rotatory power.<sup>1</sup>

*l*-(+)**Citrulline** was obtained simply by applying to *l*-(+)ornithine the excellent method lately devised by Kurtz [1938] for the preparation of *dl*-citrulline from *dl*-ornithine. This method consists in condensing the copper salt of ornithine with urea to form copper citrullinate. We have used it successfully with *l*-(+)ornithine hydrochloride prepared from gelatin by the enzymic method [Hunter, 1939].<sup>2</sup> An alternative procedure, when ornithine itself is not immediately available, is to apply Kurtz's method directly to the mixture of *l*-(+)ornithine and urea obtained by hydrolysing *l*-(+)arginine with arginase. For the success of this plan it is important that the hydrolysis of the arginine be complete, and that it be accomplished without introducing into the hydrolysate more than a minimum of extraneous material. The arginase solution used must therefore be at once purer and more potent than the ordinary crude glycerol extract of liver. One of us [Hunter, 1939] has recently described the preparation of a partly purified enzyme concentrate containing from 200 to 400 units [Hunter & Dauphinee, 1930] of arginase per ml. For the present purpose such a concentrate is entirely suitable.

The following account of one experiment among several shows the details of procedure adopted for the preparation of *l*-(+)**citrulline** from *l*-(+)arginine

<sup>1</sup> Since this paper was submitted for publication our attention has been drawn by M. Robert Duschinsky to his note (*C.R. Acad. Sci.* 1938, **207**, 735) in which are described the isolation of optically active citrulline from the water-melon and its synthesis by a method similar to our own. The figures given by M. Duschinsky for the rotation of the free amino-acid are in close agreement with those found by us.

<sup>2</sup> It is regretted that, in the discussion of the specific rotation of *l*-(+)ornithine dihydrochloride (p. 35), reference was not made to the work of Lutz & Jirgensons (*Ber. dtsch. chem. Ges.* 1931, **66**, 1221). These authors pointed out the variation of the specific rotation of the salt with concentration: they found  $[\alpha]_D^{20} + 9.7^\circ$  for  $c = 1.01$  and  $+13.3^\circ$  for  $c = 4.1$ .

hydrochloride. 10 g. of the latter salt were dissolved in 250 ml. of water, and enough *N* NaOH (3–4 ml.) was added to give a light pink colour with phenolphthalein. The solution was treated with concentrated arginase solution in such quantity as would supply 6000 units of the enzyme, and the mixture, adequately protected with toluene, was incubated at 37° for 18 hr.,<sup>1</sup> after which the Sakaguchi reaction for arginine was for all practical purposes negative. The mixture was then made acid to phenol red with acetic acid, boiled to coagulate proteins completely, filtered into a Claisen flask and evaporated *in vacuo* at 45–50° to about 50 ml. Frothing during the evaporation was controlled by addition of capryl alcohol.

The concentrated solution, containing equivalent quantities of ornithine hydrochloride and urea, was decolored with norite and filtered through a sintered glass filter. Flask and filter were washed with a few ml. of water, and the filtrate and washings brought, by dropwise addition of *N* NaOH, to pH just exceeding 7.0. The solution was then boiled, as described by Kurtz [1938], with 4 g. moist copper oxide, filtered, and, after addition of 10 g. urea, evaporated to 35 ml. After this concentrate, transferred to a tightly sealed pressure bottle, had been heated for 3 hr. in a boiling water bath, it yielded 7.4 g. of citrulline-copper (75 % of the theoretical).

The poor solubility of this product made impossible any observation of its behaviour towards polarized light: but, since it yielded a dextrorotatory citrulline (see below), and was derived from *L*-(+)-ornithine, it must have been *L*-(+)-citrulline-copper. In colour, in crystalline form and in relative insolubility in water, it did not differ in any obvious way from *dl*-citrulline-copper, as described by Wada [1930] and Kurtz [1938]. Recrystallization, which is difficult, was not attempted, and the material was probably not entirely pure. (Found: N (Kjeldahl) 19.8; Cu 15.3 %.  $(C_6H_{12}O_3N_3)_2Cu$  requires N, 20.4; Cu, 15.44 %.) The M.P. was 249°. For the purified racemic compound Wada and Ackermann each give M.P. 257–258°.

*L*-(+)-Citrulline. 5 g. of the copper complex were suspended in 125 ml. of water and decomposed with  $H_2S$ . Coagulation of colloidal copper sulphide was effected by heating with norite, and the latter was separated by centrifuging. The supernatant, still slightly coloured, was warmed again with a little norite, filtered through retentive paper into a Claisen flask, evaporated *in vacuo* to about 10 ml., transferred to an Erlenmeyer flask and treated with sufficient alcohol to produce a slight opalescence. Crystallization was started by rubbing with a glass rod, and was allowed to progress until, in about 20 min., a loose mass of crystals filled the entire liquid. Thereafter further additions of alcohol were made, about 10 ml. at a time, until during about 2 hr. 100–150 ml. had been introduced. After a night in the ice-box the crystals were collected on a glass filter, washed four or five times with 5 ml. portions of chilled alcohol and dried, first at 95° and then *in vacuo* over  $P_2O_5$ . The yield was 3.4 g., which is 80 % of the theoretical; the overall yield from the original arginine was therefore 60 %. (Found: N (Kjeldahl) 23.8 %. After recrystallization 23.9 %.  $C_6H_{13}O_3N_3$  requires N, 23.99 %.) M.P. 215–217°; after recrystallization, 219.5°. For *dl*-citrulline Wada [1933] gives 226°, Ackermann [1931] 220–222°, Kurtz [1938] 220–221°. In form the crystals were long thin prisms similar to those figured by Wada [1933].

The rotatory power of the product was measured (1) in pure aqueous solution and (2) in the presence of 1 mol. HCl. For the measurements in water

<sup>1</sup> Experience has shown that the enzyme quantities recently prescribed by one of us [Hunter, 1939] were unnecessarily large, and the time of action unnecessarily long.



we used three different preparations, A, B and C; for those with acid, preparation B and two recrystallized mixtures of various origin. All measurements were made at approximately 5% concentration and at 21–23°. The results are collected in Table I.

Table I. *Specific rotation of l-(+)-citrulline*

Sample	Mols. HCl	<i>l</i>	<i>c</i>	$\alpha$	$[\alpha]_D^{22}$ for $C_6H_{13}O_3N_3$
A	0	2.2	5.005	+ 0.38°	+ 3.45°
B	0	2.2	5.025	+ 0.37°	+ 3.35°
C	0	2.2	5.001	+ 0.40°	+ 3.64°
B	1	2.2	5.016	+ 1.97°	+ 17.9°
Recrystallized (1)	1	2.2	4.974	+ 1.96°	+ 17.9°
Recrystallized (2)	1	2.2	5.009	+ 1.98°	+ 18.0°

It will be seen that the observed difference between any two preparations hardly exceeded the experimental error, and that the rotatory power was not altered by recrystallization. There is, therefore, no reason to suppose that the material was other than pure *l*-(+)-citrulline. Its specific rotatory power in water is small—much smaller than that of the isomeric  $\alpha$ -carbamido- $\delta$ -amino-valeric acid [Hunter, 1938]—but it is greatly increased by the presence of acid. In this respect citrulline resembles the related substances arginine and ornithine and indeed the amino-acids in general.

#### SUMMARY

A procedure is described in which the method of Kurtz is applied to the preparation, from *l*-(+)-ornithine or *l*-(+)-arginine, of *l*-(+)-citrulline. The yield from arginine is about 60%. In 5% aqueous solution the substance shows  $[\alpha]_D + 3.5^\circ$ , increasing, in the presence of 1 mol. HCl, to  $+ 17.9^\circ$ .

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# XXI. STUDIES ON THE METABOLISM OF CALCIUM AND PHOSPHORUS AND ON THE AVAILABILITY OF THESE ELEMENTS FROM MILK AND FROM AN INORGANIC SOURCE

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SOME time ago experiments were reported from this laboratory [Henry & Kon, 1937, 1] on the availability to rats of Ca and P in raw and pasteurized milks. While no difference was found in this respect between the two milks two rather puzzling observations were made. In the first place, though the rats received only 60 to 70 % of the Ca and P normally retained by rats of their age on an adequate diet, they utilized only some 80 % of their intake of these minerals. Secondly, 90 % of the excreted Ca was found in the urine, a result hardly to be expected as it is usually believed that Ca is largely excreted in the faeces.

We have since carried out further experiments to determine whether this unusual excretion of Ca via the kidney and the rather low retention were due to the quality or to the quantity of the dietary Ca or possibly also to a relative shortage of P. At the same time we investigated the possibility of increasing the retention of P. It should be recalled that Ellis & Mitchell [1933] and Fairbanks & Mitchell [1936] had obtained almost 100 % retentions of Ca from diets low in Ca but carrying a generous supply of P.

In these experiments the level, ratio and source of Ca and P were varied. Milk as a source of these elements was compared with Ca phosphate. Unless otherwise stated the general experimental methods were as described in detail by Henry & Kon [1937, 1].

## PRELIMINARY EXPERIMENTS

*Experimental.*<sup>1</sup> These were exploratory experiments which dealt mainly with factors affecting the assimilation of Ca. In one experiment Ca was supplied from milk and from  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  at levels below the normal retention, in each case either with an equally subnormal level of P or with a more generous supply. In the second experiment the same plan was followed but all levels of intake of Ca and of P were raised.

1. *The diets.* To the basal low Ca and P diet of Henry & Kon [1937, 1] (diet 64, Table I) several supplements were added: (a) raw milk; (b) an equivalent quantity of diet 75, which was made from diet 64 by the addition of  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  (Table I); 18 g. of diet 75 were calculated to be equivalent in calorie, Ca and P contents to 100 ml. of the raw milk; (c) raw milk + added P, derived from  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ; (d) diet 75 + added P from the same source. This extra P was calculated to cover the urinary and faecal loss of P observed in the previous experiments [Henry & Kon, 1937, 1], which amounted to

<sup>1</sup> Read in part before the Biochemical Society, 9 October 1937 [Henry & Kon, 1937, 2].

0.21 mg. P for each ml. of milk intake. The addition did not suffice to bring the intake of P to the level normally retained by rats (Table IV). The various supplements were fed separately from the basal diet, milk in the liquid form (with or without added Na phosphate) and diet 75 mixed to a paste with  $\frac{3}{4}$  of its weight of water. When Na phosphate was added to it, the solution replaced part of the water. Table I gives the composition and analyses of the diets.

Table I. *Exps. 1 and 2. Composition and analyses of diets and milk*

	Diet 64	Diet 75	Milk*
Egg white†	14.25	13.86	—
Butter fat	5.00	4.86	—
Maize starch	68.40	66.56	—
Castor sugar	9.50	9.24	—
Ca- and P-free salt mixture‡	2.85	2.77	—
CaHPO <sub>4</sub> ·2H <sub>2</sub> O	—	2.71	—
% Ca	0.02243	0.69460	0.12540
% P	0.02585	0.54060	0.10700
% Moisture	9.96	10.20	—

\* Average of 8 bi-weekly analyses. There was very little variation between samples. The Ca and P figures are per 100 ml.

† Dried, heat-coagulated from new laid eggs.

‡ Henry & Kon [1937, 1].

18 g. of diet 75 contained 0.1252 g. Ca and 0.0973 g. P. The small discrepancy in the P content of diet 75 and of the equivalent volume of milk is explained by the fact that the P in the milk was slightly higher than anticipated. The vitamin supplements were as described elsewhere [Henry & Kon, 1937, 1].

2. *The animals.* 4 groups of 4 litter-mate male rats were used. The rats were 26–27 days old and weighed 59–71 g. at the beginning of the experiment. Litter-mates within each group were assigned by lot to the 4 treatments. They were placed in glass metabolism cages [Henry & Kon, 1937, 1]. The intakes of diet 64 and of milk or diet 75 were equalized between groups (a) and (b) and between groups (c) and (d) respectively. Table II shows that in Exp. 1 the food intakes of all 4 groups were very similar.

3. *Exp. 1.* As in previous work [Henry & Kon, 1937, 1] the milk and diet 75 were fed at such levels that the intakes of Ca and P from them were well below the normal retentions as defined by Sherman & Macleod [1925] and Sherman & Quinn [1926]. As already mentioned, groups (c) and (d) received additional P from a 6.3% solution of Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O. After a preliminary period of 3 days on the experimental diets a collection period of 14 days was started. The excreta of each group of 4 rats were pooled for analysis.

4. *Exp. 2.* When Exp. 1 was finished the amounts of milk and diet 75 were so increased as slightly to exceed the calculated normal retentions. Groups (c) and (d) again received extra P from a 6.4% solution of Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O. Otherwise the details were the same as in Exp. 1. The preliminary period lasted 4 days and was followed by a 14 days' collection period. The same rats were used in both experiments.

5. *Results.* Table II gives the food intakes, weights of the rats and the weight of faeces. The mean balance figures for Ca and P are given in Table III. Further data will be found in Table IV.

(a) *Exp. 1.* In Exp. 1 the behaviour of rats receiving milk as a source of Ca and P (group (a)) entirely confirmed the results previously reported [Henry & Kon, 1937, 1] regarding the retention of these minerals and their paths of excretion on subnormal intakes. Again only about 80% of the ingested Ca and P were retained and over 90% of the excreted Ca was in the urine. When the

Table II. *Exps. 1 and 2 (14 days each). Food intakes, weights of rats and of faeces*

Group and source of Ca and P	Food intake						Wt. of faeces† g.
	Diet 64 g.	Milk ml.	Diet 75 g.	Na <sub>2</sub> HPO <sub>4</sub> solution ml.	Wt. of rats, g.		
					Initial	Final	
Exp. 1							
(a) Milk	106.13	164.99	--	---	67.5	107.7	2.49
(b) Diet 75	106.11	---	29.69	---	65.8	106.0	2.16
(c) Milk + Na <sub>2</sub> HPO <sub>4</sub>	108.68	165.57	--	6.619	67.5	109.2	2.62
(d) Diet 75 + Na <sub>2</sub> HPO <sub>4</sub>	108.68	---	29.75	6.614	67.8	107.8	2.31
Exp. 2							
(a) Milk	107.32	293.48	---	---	117.0	150.8	3.80
(b) Diet 75	107.32	---	52.76	---	115.8	149.5	2.92
(c) Milk + Na <sub>2</sub> HPO <sub>4</sub> (i)*	100.69	324.75	-	12.99	121.3	151.0	3.87
Milk + Na <sub>2</sub> HPO <sub>4</sub> (ii)†	105.82	315.33	---	12.61	116.0	151.3	---
(d) Diet 75 + Na <sub>2</sub> HPO <sub>4</sub> (i)*	101.00	---	58.34	12.96	119.3	153.8	2.84
Diet 75 + Na <sub>2</sub> HPO <sub>4</sub> (ii)†	105.67	---	56.61	12.58	115.7	156.3	---

\* (i) = Average for all 4 rats.

† (ii) = Average for 3 rats, omitting the rats mentioned in the text, p. 176.

‡ Air dry.

Table III. *Exps. 1 and 2 (14 days each). Mean balance figures for Ca and P*

Group	Ca intake g.	Ca excretion, g.		Ca balance g.	P intake g.	P excretion, g.		P balance g.
		Urine	Faeces			Urine	Faeces	
Exp. 1								
(a)	0.2306	0.0486	0.0041	0.1779	0.2038	0.0129	0.0232	0.1677
(b)	0.2300	0.0691	0.0280	0.1329	0.1879	0.0040	0.0373	0.1466
(c)	0.2321	0.0315	0.0031	0.1976	0.2415	0.0351	0.0253	0.1811
(d)	0.2311	0.0369	0.0225	0.1717	0.2251	0.0240	0.0371	0.1640
Exp. 2								
(a)	0.3934	0.0103	0.0145	0.3686	0.3420	0.0676	0.0302	0.2442
(b)	0.3906	0.0085	0.0380	0.3441	0.3129	0.0297	0.0550	0.2282
(c) (i)*	0.4317	0.0085	0.0170	0.4062	0.4468	0.1536	0.0356	0.2576
(c) (ii)*	0.4207	—	—	—	0.4352	—	—	—
(d) (i)*	0.4279	0.0052	0.0478	0.3749	0.4136	0.1119	0.0607	0.2410
(d) (ii)*	0.4169	—	—	—	0.4033	—	—	—

\* Cf. footnotes, Table II.

minerals were derived from Ca phosphate (group (b)) the results were similar but the retentions (especially of Ca) were lower and more Ca was excreted in the faeces, reducing the urinary excretion to 71 % of the total. It should be remembered that the two groups (a) and (b) were not strictly comparable owing to the lower P intake of the latter group. Moreover, it is quite obvious from further experiments (pp. 176 and 184) that at low levels of intake the retention of Ca is markedly affected by the quantity of P in the diet. Further, milk and diet 75 differed with regard to buffering power and acid-base balance. These remarks apply equally to groups (c) and (d) and to all four groups in Exp. 2.

The addition of Na acid phosphate to the milk diet (group (c)) brought the P intake to 90 % of the normal retention and increased the retention of Ca to 85 % by considerably lowering the urinary excretion. The absolute retention of P also improved, though more was lost in the urine. The additional P had a similar effect on the rats receiving the Ca phosphate diet but the retentions of Ca (and of P), though increased, were still inferior to the retentions on the milk diet.

Table IV. *Exps. 1 and 2 (14 days each). Various data for Ca and P retentions and excretions*

Group	% Ca retention	Ca				% P retention	P				% total P excretion in the urine
		% Ca intake derived from milk or diet 75	intake mg./g. gain in wt.	% of normal retention at same rate of intake*	% total Ca excretion in the urine		% P intake derived from milk or diet 75	intake mg./g. gain in wt.	% of normal retention at same rate of intake†		
Exp. 1											
(a)	77.14	89.7	5.74	72.1	92.31	82.30	86.5	5.07	78.4	35.83	
(b)	57.77	89.6	5.72	71.9	71.12	78.01	85.6	4.67	71.1	9.74	
(c)	85.12	88.7	5.56	70.9	91.09	74.97	73.4	5.79	89.3	58.17	
(d)	74.28	89.4	5.78	72.2	62.14	72.85	71.5	5.63	86.5	39.32	
Exp. 2											
(a)	93.70	93.9	11.63	112.4	41.73	71.42	91.9	10.12	148.6	69.10	
(b)	88.08	93.9	11.60	111.5	18.31	72.93	91.1	9.28	142.2	35.03	
(c) (i)‡	94.09	94.8	14.53	139.3	33.40	57.67	78.0	15.04	223.4	81.18	
(c) (ii)‡	—	—	11.91	116.9	—	—	—	12.33	185.2	—	
(d) (i)‡	87.61	94.7	12.40	122.3	9.81	58.27	76.3	11.99	179.8	64.81	
(d) (ii)‡	—	—	10.22	104.0	—	—	—	9.88	152.2	—	

\* Calculated from data of Sherman &amp; Macleod [1925].

† Calculated from data of Sherman &amp; Quinn [1926].

‡ Cf. footnotes, Table II.

(b) *Exp. 2.* In Exp. 2 it was aimed to supply to groups (a) and (b) quantities of Ca and P just above the normal retentions. Table IV shows that this was satisfactorily achieved for Ca in that the amounts fed surpassed the normal retention figures by about 12%, but that for P the margin was over 40%. This is explained by differences in the shape of the curves relating Ca and P retentions to the weight of the rat [Sherman & Macleod, 1925; Sherman & Quinn, 1926]. In groups (c) and (d) the Ca intakes were relatively higher (some 40 and 20% respectively above the normal) owing to the poor growth of one rat in each group. One rat in group (c) refused food during the last week and the intake of his litter-mate in group (d) had to be cut down accordingly. The marked discrepancy in the P intakes of these groups expressed as % normal retention (Table IV) was due to smaller gain in weight and to higher absolute P intake of one group. When the two unsatisfactory rats are omitted the average Ca and P intakes for the groups drop down to 117 and 185% and 104 and 152% respectively of the normal retentions.

The Ca intake in Exp. 2 was almost twice that in Exp. 1 (Table III), yet, in the presence of a relative surplus of P, the total excretion of Ca decreased because of a marked reduction in the urinary loss, so that more than twice as much Ca was retained and the efficiency of utilization rose to 94% for milk and 88% for Ca phosphate (Table IV). Further addition of P in groups (c) and (d) did not increase the % retention. There was a slight increase in the faecal loss of Ca in groups (a) and (b) in this experiment as compared with groups (c) and (d) of Exp. 1, but it should be pointed out that the shift in the % excretion of Ca from urine to faeces was chiefly due to the decrease of the urinary output.

It is probable that the retention of 94% of milk Ca was not maximal and that it might have been higher if Ca had been given at a level slightly below instead of above that of the calculated normal retentions. The increase in faecal Ca (Table III) supports this view as it indicates that excess unabsorbed Ca was passing through the gut.

The results of the two preliminary experiments throw light on some of the peculiarities noticed in our earlier work [Henry & Kon, 1937, 1]. They do not explain why P was not quantitatively retained, but the relatively low retention of Ca must have been due to an actual shortage of P as it was greatly improved by an increase in the level of P. The Ca of milk appears to be almost completely digestible and to be taken up in the blood stream. In the absence of sufficient P a part of it cannot be retained and is lost to the body through the urine. Hence much more Ca is excreted under these conditions through the kidney than through the gut. The Ca from an inorganic source behaves similarly but appears to be less digestible, since in all comparisons more was found in the faeces. Even then, under certain conditions, over 70 % of it was excreted in the urine. On the Ca phosphate diets more P was also excreted in the faeces and when excretions on these diets are compared with excretions on the milk diets it is found that the excess faecal Ca and P are in the ratios of 1.70 : 1, 1.64 : 1, 0.95 : 1 and 1.23 : 1 respectively for the several milk-Ca phosphate comparisons (Table III), indicating the passage of unchanged  $\text{CaHPO}_4$  through the gut. As already stated (p. 175) the milk and Ca phosphate diets were not strictly comparable. The problem of availability of Ca and P from different sources and the question of the metabolism of P were therefore further studied in the following experiments.

### EXPERIMENT 3<sup>1</sup> (MAIN EXPERIMENT)

1. *The diets.* Two alterations were made in the composition of the diets. In the first instance liquid raw milk fed separately was replaced by spray-dried milk incorporated in the ration. This greatly facilitated the equalization of mineral intakes between milk and Ca phosphate diets which could be arranged beforehand with certainty. In addition it yielded information on the effect of drying on the availability of milk minerals. The second alteration was in the make-up of the substitute diet carrying the inorganic Ca phosphate. An attempt was made to render it equivalent to milk with respect not only to calorie, Ca and P contents but also to the relative proportions of constituents and to acid-base balance. A new diet was added to study the effect on the retention of P of excess Ca and of subnormal P intakes.

(a) *The basal diets.* The following three basal diets were used: (i) A diet low in both Ca and P, identical with diet 64 of the preliminary experiments (p. 173). (ii) A diet low in Ca but relatively high in P. It was made from diet 64 by adding to it sufficient  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  to raise the P content of the diet to 0.13–0.15 %. This level was calculated from the preliminary experiments (p. 176) and from our previous work [Henry & Kon, 1937, 1] to supply, together with the milk or substitute diet, an excess of P over the normal retentions. (iii) A diet relatively high in Ca but low in P. This was obtained by adding  $\text{CaCO}_3$  to the low Ca and P diet to bring the Ca content to 0.20–0.21 %. Table V gives the analyses of these basal diets.

Table V. *Exp. 3. Ca, P and moisture contents of the basal diets*

Basal diet no.	% Ca	% P	% moisture
(i)	0.0195	0.0239	10.43
(ii)	0.0200	0.1489	10.91
(iii)	0.2170	0.0239	10.36

To each of these basal diets suboptimal amounts of Ca and P were added either from milk or from  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ . This was done by mixing with the diets requisite quantities of spray-dried milk or of the substitute diet.

<sup>1</sup> Read in part before the Biochemical Society, 8 October 1938 [Henry & Kon, 1938].

(b) *The substitute diet.* The diet was planned to be equicaloric with the spray-dried milk, to have a similar acid-base balance and to resemble it regarding the protein, fat and lactose contents. As the Ca and P were to be given in inorganic combination as Ca phosphate, casein and lactalbumin were replaced by egg-white. Similarly the salts had to be altered. We are indebted to Dr W. L. Davies for advice on this matter. Information on the salt combinations in milk was taken from his book [Davies, 1936]. The composition of the diet is given in Table VI. It was prepared without the addition of  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and analysed for Ca and P. The calculated amount of Ca phosphate was blended directly with the basal diet and the rest of the substitute diet was then mixed in. For this reason Ca and P figures are not given in Table VI but Table VIII shows that the quantities of these elements in the substitute diet and in milk were practically identical.

(c) *The spray-dried milk.* The sample was dried by Messrs Aplin and Barrett at their Frome Factory in a Kestner plant after preheating. It was stored in sealed tins under  $\text{N}_2$  and  $\text{CO}_2$  and was 3 months old when used. The composition of the milk is given in Table VI.

Table VI. *Exp. 3. Composition of the substitute diet and of spray-dried milk*

	Substitute diet (as mixed) %	Dried milk %
Butter-fat	28.73	28.97
Egg white	27.63	—
Protein ( $\text{N} \times 6.38$ )	—	25.94
Lactose	36.57	36.00
NaCl	0.402	—
KCl	0.342	—
K citrate	1.948	—
Mg citrate	0.689	—
$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	3.682	—
Ca	—	0.9454
P	—	0.7307
Moisture	2.67	1.1

(d) *The final diets.* These were mixed in the proportion of 100 g. dried milk or 101.4 g. substitute diet to 569.56 g. of each basal diet. The slight difference

Table VII. *Exp. 3. Composition of the final diets*

Basal diets	...	...	(i) Low Ca and low P		(ii) Low Ca and high P		(iii) High Ca and low P	
			Milk	" $\text{CaHPO}_4$ "	Milk	" $\text{CaHPO}_4$ "	Milk	" $\text{CaHPO}_4$ "
Supplements	...	...	89	90	91	92	93	94
Final diet no.	...	...	89	90	91	92	93	94
Components:								
Egg white*			12.12	16.27	11.94	16.09	12.06	16.21
Butter fat			4.25	8.59	4.19	8.53	4.23	8.57
Maize starch			58.18	58.05	57.32	57.19	57.90	57.76
Castor sugar			8.08	8.06	7.96	7.94	8.04	8.02
Lactose			—	5.53	—	5.53	—	5.53
Ca- and P-free salt mixture†			2.424	2.419	2.389	2.383	2.412	2.407
NaCl			—	0.061	—	0.061	—	0.061
KCl			—	0.052	—	0.052	—	0.052
K citrate			—	0.295	—	0.295	—	0.295
Mg citrate			—	0.104	—	0.104	—	0.104
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$			—	—	1.260	1.257	—	—
$\text{CaCO}_3$			—	—	—	—	0.423	0.422
Spray dried whole milk			14.94	—	14.94	—	14.94	—
$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$			—	0.557	—	0.557	—	0.557

\* Dried, heat-coagulated from new laid eggs.

† Henry & Kon [1937, 1].

in quantities between milk and substitute diets was due to difference in moisture content. The ratios in which the diets were mixed were calculated from the relative intakes of milk and basal diet observed in our previous experiments [cf. Table II and Henry & Kon, 1937, 1].

Table VII gives the composition and Table VIII the analyses of the final diets.

Table VIII. *Exp. 3. Analyses of experimental diets*

Diet no.	% Ca	% P	% moisture	Ca : P	% Ca derived from milk or Ca phosphate	% P derived from milk or Ca phosphate	% of normal retention supplied*	
					Ca	P		
89	0.1610	0.1284	9.36	1.25	89.5	84.3	65.1	68.5
90	0.1610	0.1264	9.41	1.27	89.5	84.3		
91	0.1600	0.2367	9.95	0.68	89.3	46.3	63.8	125.5
92	0.1607	0.2328	10.01	0.68	89.3	46.3		
93	0.3284	0.1283	9.36	2.56	43.3	84.3	154.5	74.7
94	0.3280	0.1271	9.39	2.58	43.5	84.3		

\* These figures were calculated after the experiment was completed from data of Sherman & Macleod [1925] and Sherman & Quinn [1926].

2. *The animals.* Six litters were used, each consisting of 6 male rats 23–24 days old and weighing 42–56 g. The animals were distributed by lot but in cases where differences in weight between litter-mates were appreciable uniform pairs were first selected for the milk-Ca phosphate comparisons and the final allocation was by toss of coin. The animals were numbered consecutively, rats 1–6, 7–12 etc. being litter-mates. The two types of metabolic cages used and the methods of feeding, giving vitamin additions, collecting excreta etc. were as already described [Henry & Kon, 1937, 1]. Distilled water was given for drinking. The experimental diets were fed for 4 days before the collection of excreta was started. The experiment lasted 5 weeks, the cages being washed once a week. At the end of the experiment the rats were killed by coal gas and the carcasses, after removal of the intestinal contents, were ashed and analysed for Ca and P. In ashing, 20 ml. of a 10% solution of Na acetate were added to supply fixed base, as without this precaution some loss of P occurred in our earlier experiments [Henry & Kon, 1937, 1].

3. *Results.* The results of this experiment are set out in Tables IX–XIII. Table IX gives the individual and mean data for the weights of rats and faeces; Tables X and XI give various data for individual rats concerning the intakes, excretions and retentions of Ca and of P. Table XII gives group means of data calculated from the two previous tables. Table XIII contains the results of statistical tests of significance of some of the differences. For this purpose the paired *t*-test of "Student" [1908; 1925] was used.

Towards the end of the experiment rat no. 34 on diet 92 showed a definite loss of appetite and passed blood in the faeces. During the last few days, therefore, no attempt was made to equalize the food intakes of this rat and of its litter-mate, no. 33, on diet 91. When the animals were killed for analysis it was found that the intestinal contents of rat 34 were, owing to the presence of blood, of a chocolate colour instead of the normal black. This was most probably due to internal injury caused by the animal nibbling off part of a metal tag on the cage. As can be seen from Table X the faeces of this animal contained abnormally large quantities of Ca and P, doubtless due to the haemorrhage. The figures for this rat (and for its litter-mates when necessary for comparative purposes) have been omitted from certain means.



Table IX. *Exp. 3 (35 days). Individual and mean data for the weights of rats and of faeces*

Rat no.	Wt. of rat, g.		Wt. of faeces* g.	Rat no.	Wt. of rat, g.		Wt. of faeces* g.
	Initial	Final			Initial	Final	
Diet 89				Diet 90			
1	62	180	6.27	2	60	174	8.12
7	56	135	4.32	8	56	131	5.29
13	53	156	5.65	14	55	158	6.07
19	64	148	5.15	20	63	148	5.23
25	55	138	4.57	26	50	140	4.70
31	56	168	6.22	32	55	162	5.69
Mean (i)†	57.7	154.2	5.36		56.5	152.2	5.85
Mean (ii)‡	—	—	—		56.8	150.2	5.88
Diet 91				Diet 92			
3	63	166	5.22	4	62	161	6.12
9	62	148	4.65	10	62	149	5.20
15	60	155	5.37	16	60	146	5.38
21	63	149	4.82	22	63	153	5.12
27	60	167	5.00	28	57	164	5.58
33	59	137	4.68	34	57	124	8.25
Mean (i)†	61.2	153.7	4.96		—	—	—
Mean (ii)‡	61.6	157.0	5.01		60.8	154.6	5.48
Diet 93				Diet 94			
5	64	159	5.53	6	61	148	6.68
11	55	129	5.90	12	55	125	6.23
17	53	127	4.60	18	54	124	4.68
23	59	142	4.92	24	58	138	5.82
29	51	130	5.06	30	50	136	5.41
35	51	115	6.32	36	55	120	5.76
Mean (i)†	55.5	133.7	5.39		55.5	131.8	5.76
Mean (ii)‡	—	—	—		55.6	134.2	5.76

\* Air-dry.

† Mean (i) = Mean for 6 animals.

‡ Mean (ii) = Mean for 5 animals, omitting litter VI (rats 31-36 incl.).

Table X. *Exp. 3 (35 days). Individual figures for the intakes and excretions of Ca and P*

Rat no.	Diet intake g.	Ca intake g.	Ca excretion, g.		P intake g.	P excretion, g.	
			Urine	Faeces		Urine	Faeces
			Diet 89				
1	377.93	0.6085	0.1293	0.0038	0.4853	0.0069	0.0577
7	286.88	0.4619	0.0763	0.0045	0.3684	0.0120	0.0341
13	359.96	0.5795	0.0901	0.0049	0.4622	0.0113	0.0459
19	332.99	0.5361	0.0888	0.0072	0.4276	0.0236	0.0462
25	306.27	0.4931	0.0795	0.0048	0.3933	0.0118	0.0399
31	386.46	0.6222	0.1175	0.0031	0.4962	0.0142	0.0534
Diet 90							
2	377.70	0.6081	0.1541	0.0181	0.4774	0.0046	0.0842
8	288.00	0.4637	0.1008	0.0100	0.3640	0.0090	0.0528
14	359.59	0.5789	0.1068	0.0195	0.4545	0.0081	0.0582
20	331.17	0.5332	0.0860	0.0167	0.4186	0.0099	0.0562
26	306.07	0.4928	0.0946	0.0249	0.3869	0.0039	0.0498
32	385.33	0.6204	0.0971	0.0277	0.4871	0.0136	0.0553

Table X (cont.)

Rat no.	Diet intake g.	Ca intake g.	Ca excretion, g.		P intake g.	P excretion, g.	
			Urine	Faeces		Urine	Faeces
Diet 91							
3	333.33	0.5333	0.0101	0.0027	0.7890	0.3039	0.0501
9	314.50	0.5032	0.0089	0.0027	0.7444	0.3029	0.0483
15	328.90	0.5262	0.0020	0.0038	0.7785	0.3065	0.0530
21	328.43	0.5255	0.0043	0.0037	0.7774	0.3299	0.0493
27	365.83	0.5853	0.0085	0.0036	0.8659	0.3673	0.0489
33	313.92	0.5023	0.0066	0.0041	0.7430	0.3329	0.0499
Diet 92							
4	334.48	0.5375	0.0077	0.0116	0.7787	0.2991	0.0692
10	314.10	0.5048	0.0021	0.0119	0.7312	0.3018	0.0577
16	328.33	0.5276	0.0017	0.0163	0.7644	0.3189	0.0636
22	328.11	0.5273	0.0060	0.0174	0.7638	0.3142	0.0633
28	365.75	0.5878	0.0076	0.0116	0.8515	0.3443	0.0628
34	297.62	0.4783	0.0102	0.2082	0.6929	0.2543	0.1171
Diet 93							
5	337.19	1.1073	0.4784	0.0993	0.4326	0.0048	0.0350
11	290.41	0.9537	0.2964	0.1931	0.3726	0.0045	0.0370
17	292.77	0.9615	0.2993	0.1646	0.3756	0.0052	0.0322
23	323.52	1.0624	0.3896	0.1222	0.4151	0.0063	0.0385
29	288.87	0.9486	0.3506	0.1235	0.3706	0.0056	0.0417
35	277.78	0.9122	0.2856	0.1852	0.3564	0.0056	0.0543
Diet 94							
6	336.08	1.1023	0.4344	0.2035	0.4272	0.0062	0.0774
12	290.12	0.9516	0.3328	0.2330	0.3687	0.0054	0.0836
18	292.13	0.9582	0.3181	0.1800	0.3713	0.0067	0.0574
24	322.33	1.0572	0.3297	0.2475	0.4097	0.0056	0.0720
30	288.00	0.9446	0.3721	0.1942	0.3660	0.0044	0.0683
36	276.80	0.9079	0.4429	0.0956	0.3518	0.0038	0.0572

Table XI. *Exp. 3 (35 days). Individual figures for the Ca and P contents of the rat carcasses*

Rat no.	Wt. of carcass g.	Ca in carcass g.	Esti- mated de- posited	P in	Esti- mated de- posited	Rat no.	Wt. of carcass g.	Ca in carcass g.	Esti- mated de- posited	P in	Esti- mated de- posited
			Ca, g.*	g.	P, g.*				Ca, g.*	g.	P, g.*
Diet 89						Diet 90					
1	178	0.8346	0.4752	0.7243	0.4203	2	171	0.7624	0.4146	0.6764	0.3822
7	133	0.7471	0.4107	0.6151	0.3464	8	129	0.7084	0.3720	0.5972	0.3285
13	154	0.7572	0.4762	0.6504	0.4102	14	153	0.7591	0.4675	0.6453	0.3960
19	145	0.8297	0.4236	0.6653	0.3477	20	147	0.8128	0.4130	0.6590	0.3464
25	133	0.7377	0.4262	0.6082	0.3455	26	134	0.6935	0.4103	0.5790	0.3401
31	165	0.8808	0.5067	0.7337	0.4567	32	157	0.8410	0.4735	0.6922	0.4201
Diet 91						Diet 92					
3	162	0.8840	—	0.7360	—	4	157	0.8793	—	0.7221	—
9	144	0.8685	—	0.6784	—	10	145	0.8588	—	0.6814	—
15	151	0.8412	—	0.6844	—	16	142	0.8251	—	0.6605	—
21	147	0.9165	—	0.7098	—	22	149	0.9045	—	0.6999	—
27	162	0.9161	—	0.7302	—	28	158	0.8914	—	0.7167	—
33	131	0.8858	—	0.6521	—	34	120	0.7909	—	0.5948	—
Diet 93						Diet 94					
5	158	0.8765	0.5055	0.6868	0.3730	6	144	0.7883	0.4347	0.6247	0.3256
11	123	0.8084	0.4780	0.6112	0.3473	12	120	0.7047	0.3743	0.5500	0.2861
17	120	0.8015	0.5205	0.6000	0.3598	18	120	0.7494	0.4631	0.5693	0.3245
23	138	0.9123	0.5379	0.6724	0.3796	24	133	0.8350	0.4669	0.6345	0.3467
29	125	0.7757	0.4869	0.5790	0.3354	30	130	0.6492	0.3660	0.5458	0.3069
35	112	0.8146	0.4739	0.5845	0.3322	36	116	0.7558	0.3883	0.5711	0.2990

\* Cf. p. 183.

Table XII. *Exp. 3 (35 days)*(a) *Group means calculated from data in Table X*

Diet no.	Diet intake g.	Ca intake g.	Ca excretion, g. Urine      Faeces	Ca balance g.	% Ca retention	% total Ca excretion in urine	P intake g.	P excretion, g. Urine      Faeces	P balance g.	% P retention	% total P excretion in urine
89 (i)*	341.75	0.5502	0.0969	0.0047	0.4486	95.11	0.4388	0.0132	0.3793	86.45	22.35
90 (i)*	341.31	0.5495	0.1066	0.0195	0.4235	84.29	0.4314	0.0082	0.3638	84.34	12.32
90 (ii)*	332.51	0.5353	0.1085	0.0178	0.4090	85.58	0.4203	0.0071	0.3529	84.03	10.84
91 (ii)*	334.20	0.5347	0.0068	0.0033	0.5246	62.82	0.7910	0.3221	0.4190	52.98	86.52
92 (ii)*	334.16	0.5370	0.0050	0.0138	0.5182	25.91	0.7779	0.3157	0.3989	51.25	83.27
91 (i)*	330.82	0.5293	0.0067	0.0035	0.5191	62.63	0.7830	0.3239	0.4092	52.23	86.59
93 (i)*	301.76	0.9910	0.3500	0.1480	0.4930	69.77	0.3871	0.0033	0.3420	88.22	12.01
94 (i)*	300.91	0.9870	0.3717	0.1923	0.4230	65.98	0.3825	0.0053	0.3078	80.48	7.24
94 (ii)*	305.93	1.0028	0.3574	0.2117	0.4337	62.72	0.3886	0.0057	0.3112	80.05	7.44

(b) *Group means calculated from data in Table XI*

Diet no.	Wt. of carcass g.	Ca in carcass g.      %	Estimated† initial Ca, g.	Ca deposited g.	P in carcass g.      %	Estimated† initial P, g.	P deposited g.	Ca : P ratio of carcass
89 (i)*	151.3	0.7979	0.3305	0.4531	0.6662	0.2784	0.3878	1.197
90 (i)*	148.5	0.7629	0.5162	0.4252	0.6415	0.2726	0.3689	1.190
90 (ii)*	146.8	0.7472	0.5123	0.4155	0.6314	0.2728	0.3586	1.186
91 (ii)*	153.2	0.8853	0.5790	—	0.7078	0.2888	—	1.250
92 (ii)*	150.2	0.8718	0.5809	—	0.6961	0.2972	—	1.252
91 (i)*	140.5	0.8854	0.5952	—	0.6985	0.2893	—	1.268
93 (i)*	129.3	0.8315	0.6481	0.5005	0.6223	0.2678	0.3545	1.338
94 (i)*	127.2	0.7471	0.5897	0.4156	0.5826	0.2678	0.3148	1.282
94 (ii)*	129.4	0.7453	0.5773	0.3243	0.5849	0.2669	0.3180	1.274

\* Cf. footnote to Table IX.

† Cf. p. 183.

Table XIII. *Exp. 3. Statistical treatment of some of the differences*

Dietary difference tested*	Calcium			Phosphorus		
	Difference	Standard error of the mean	P†	Difference	Standard error of the mean	P†
(a) % retention at different levels of Ca and P intake						
91-89	+16.44	±0.84	1 : over 11,000, signif.	—	—	—
92-90	+20.01	±1.64	1 : 3570, signif.	—	—	—
93-89	—	—	—	+1.77	±1.25	1 : 5, not signif.
94-90	—	—	—	-3.86	±0.93	1 : 111, signif.
(b) % retention from milk or CaHPO <sub>4</sub>						
89-90	+4.58	±1.15	1 : 96, signif.	+2.11	±0.95	1 : 13, not signif.
91-92	+1.62	±0.44	1 : 47, signif.	+1.73	±0.72	1 : 14, not signif.
93-94	+6.94	±0.88	1 : 1850, signif.	+7.74	±1.71	1 : 161, signif.
(c) Faecal excretion from milk or CaHPO <sub>4</sub>						
90-89	+0.0148	±0.0028	1 : 312, signif.	+0.0132	±0.0034	1 : 86, signif.
92-91	+0.0105	±0.0011	1 : 1430, signif.	+0.0134	±0.0017	1 : 735, signif.
94-93	+0.0443	±0.0314	1 : 5, not signif.	+0.0295	±0.0063	1 : 184, signif.
(d) Urinary excretion from milk or CaHPO <sub>4</sub>						
90-89	-0.0097	±0.0073	1 : 4, not signif.	-0.0050	±0.0020	1 : 18, not signif.
92-91	-0.0018	±0.0014	1 : 4, not signif.	-0.0064	±0.0061	1 : 3, not signif.
94-93	+0.0217	±0.0314	1 : 2, not signif.	0.0000	±0.0006	Not signif.

\* The figures in this column refer to the numbers of the diets.

† P - probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

The two diets containing additional Ca, nos. 93 and 94, appeared to be less palatable than the other diets. Less was eaten, there was a good deal of scattering and the animals grew less well on these diets (Tables IX, X and XIIa). Though great care was taken in collecting the scattered diet as soon as possible, some contamination with urine could not be avoided. Such diet was then carefully dried by pressing between filter papers. These were then extracted with dilute HNO<sub>3</sub> and the washings were added to the urines. That this procedure was satisfactory and that the analytical results for groups 93 and 94 were correct can be shown in the following way. The animals receiving diets 91 and 92 did not scatter the food at all. It was therefore possible to calculate their initial stores of Ca and P from the analyses of their carcasses at the end of the experiment and from their Ca and P balances. These figures were then used to estimate [Henry & Kon, 1937, 1] the initial mineral stores of their litter-mates in groups 89 and 90, and 93 and 94. From these data and the carcass analyses the Ca and P depositions in these rats were calculated. Table XIIa and b shows that the agreement between figures obtained in this way and the Ca and P balances of these rats is very good, especially as no litter-mates were actually analysed at the beginning of the experiment. These results justify confidence in the use of the urinary analyses for groups 93 and 94.

Turning now to a discussion of the results it is convenient to review them under two headings. The effects of changing the levels of Ca and P will be considered first and the differences in availability of Ca and P according to source will be dealt with later.

(a) *Effect of level of Ca and P in the diet.* When Ca and P were both supplied in quantities well below the normal retentions (diets 89 and 90) the results (Tables X and XIIa) were very similar to those obtained in the previous experiments in this paper (p. 174) and in earlier work [Henry & Kon, 1937, 1].

Again the retention of Ca was about 80 %, and some 90 % of the excreted Ca was found in the urine. The retention of P, amounting to some 85 %, was also not quantitative. In these respects it mattered little whether the minerals were derived from milk or from an inorganic source, there was no qualitative difference in their behaviour. It should be noted that there was a closer agreement between the milk and non-milk diets than in the preliminary experiments (p. 175), most probably because the two types of diets were this time more strictly comparable.

The most dramatic change followed the increase in dietary P (Tables X and XIIa). This brought about a very marked increase in the retention of Ca, with the result that the % retention rose to 98.1 and 96.5 respectively for the two types of diets. Table XIIIa shows the high statistical significance of this change. Our supposition that the originally low retention of Ca was due to a shortage of P was thus definitely confirmed. The excretion of Ca per g. Ca intake decreased almost tenfold. This was almost entirely due to a diminished excretion of Ca in the urine, the fall amounting to some 160–180 mg. per g. Ca intake or about 93–95 % of the original urinary Ca. The faecal Ca also fell by some 30 and 27 % respectively, but this amounted to only 3–10 mg. per g. Ca intake, as can be calculated from data given in Tables X and XIIa. As a result a higher proportion of the small quantity of Ca still lost on these diets was found in the faeces; this is specially noticeable on the Ca phosphate diet. The increased retention of Ca was obviously accompanied by increased deposition of P, 567 mg. and 488 mg. of P respectively being deposited per g. increase in Ca deposition.

The increase in dietary Ca, the P being left unchanged (diets 93 and 94), had a much smaller effect on the metabolism of P. The % retention increased slightly but not significantly for the milk diet (Tables X, XIIa and XIIIa) but dropped significantly, though not to a marked extent, for the Ca phosphate diet. This latter effect was due to increased faecal loss (Tables X and XIIa). In both cases the urinary excretion was diminished.

(b) *The availability of Ca and P from milk and from calcium phosphate.* In all three comparisons the rats retained more Ca and more P from milk than from the inorganic source (Tables X and XIIa). The differences are more pronounced for Ca and Table XIIIb shows that in every case the superiority of milk is statistically significant.<sup>1</sup> Under conditions leading to maximal retention of Ca (diets 91 and 92) the Ca from Ca phosphate is retained very efficiently, all but 3.5 % being deposited in the body. This is only 1.6 % less than the retention from milk and it is interesting that such a small difference should be statistically significant. This is probably because of the internal consistency of the results yielded by the two groups of rats, which can be calculated from Table X.

Of the differences in P retention only one, that between diets 93 and 94, is statistically significant; the others, though suggestive with odds of 13:1 and 14:1 against the results being due to chance, are not. The differences in availability of minerals between the milk and non-milk diets are mostly due to higher faecal excretions of both Ca and P on the Ca phosphate diet. These differences in faecal excretions are all statistically significant (Table XIIIc) with the exception of the comparison of diets 93 and 94 in respect of Ca. The urinary excretions differ less and not always in the same direction. They are greater for

<sup>1</sup> Strictly speaking only 90 % of the Ca was derived from milk or Ca phosphate. The remaining 10 % from the basal diet was common to both treatments. A correction to this effect might be introduced in the calculations as has been done by Ellis & Mitchell [1933]. For reasons already given [Henry & Kon, 1937, 1] we prefer not to do this.

milk rats in one case for Ca (diets 91 and 92) and in two cases for P. Table XIII*d* shows that these differences are not significant. The faecal loss of both minerals is invariably higher on the Ca phosphate diet and the Ca : P ratio of the excess excretions suggests that the poorer availability of the inorganic additions was due to non-absorption of some of the Ca phosphate which passed unchanged through the gut. The ratios are 1.12 : 1, 0.78 : 1 and 1.50 : 1 respectively for the three comparisons. The Ca : P ratio of  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  is 1.29 : 1.

## DISCUSSION

### 1. *The metabolism of Ca and P*

Most of the Ca excreted by adult animals on normal diets is found in the faeces and until recently it was commonly believed that the gut functions as an excretory organ for this element. It was thought that Ca was absorbed by the small intestine and re-excreted in the lower bowel. A summary of relevant work will be found in the reviews of Stewart & Percival [1928] and of Hunter [1930]. More recently, however, Nicolaysen [1934] and Christiansen [1936] came to the conclusion that much of the evidence brought forward in favour of an active excretion of Ca by the gut was either experimentally unsatisfactory or inconclusive. Their own experiments on dogs [Nicolaysen, 1934] or rats [Nicolaysen, 1937], and on rabbits and goats [Christiansen, 1936], did not support the view that the lower bowel excretes Ca. Recently also the careful observations of Welch *et al.* [1936] and of Johnson [1937] have shown that in man only negligible quantities of Ca are excreted by the colon. Wright *et al.* [1938] have come to a similar conclusion regarding the cat. On the other hand Cowell [1937] has produced experimental arguments for the excretion of Ca into the large intestine of the rabbit which cannot be lightly dismissed and Dr Quinn of the Onderstepoort Laboratory, who is studying the digestive processes of sheep by means of fistulae at several intestinal levels, is of the opinion that the possibility of active excretion of Ca in the colon of ruminants must be seriously considered [private communication]. It is quite possible that herbivora behave differently in this respect from omnivorous or carnivorous animals. For both the latter the recent evidence speaks strongly against the active participation of the colon in Ca metabolism and in our opinion our work on young growing rats lends definite support to this view. In the first instance we find that on diets supplying a sufficiency of P but not enough Ca for proper calcification, the latter mineral is most efficiently retained, the daily loss in the period of rapid growth amounting to only 0.2 mg. daily in the urine and 0.1 mg. in the faeces (Table XII*a*, diet 91). Under such conditions there can be no doubt that there is no significant excretion of Ca through the colon. But even when the supply of P is so reduced that the food Ca, though limited in amount, cannot be efficiently deposited in the bone and some of it must be excreted, this excretion takes place almost exclusively through the urinary path. The daily loss amounts in this case to 2.8 mg. in the urine but still to only 0.1 mg. in the faeces (Table XII*a*, diet 89). Admittedly these very low faecal excretions are found only on the diets in which milk supplies most of the Ca. When this is derived from Ca phosphate the daily losses of Ca in the faeces rise to 0.4 mg. (diet 92, Table XII*a*) and 0.6 mg. (diet 90, Table XII*a*) respectively, but the faecal P is correspondingly increased and, in all probability, the higher faecal Ca on these diets does not indicate any active excretion of Ca through the large intestine but only that a part of the Ca phosphate has passed through the gut unchanged. In this case the difference in the urinary loss of Ca is even more spectacular, 0.14 mg. daily for the diet

containing adequate P (diet 92, Table XIIa) and more than 20 times this amount, 3.0 mg., daily for the low-P diet (diet 90, Table XIIa).

The efficiency with which growing rats are able, under conditions favouring the uptake of Ca, to retain this element has already been pointed out by Ellis & Mitchell [1933]. These authors expressed the view that "in the growing rat and possibly in all growing animals there is no integral requirement of Ca for maintenance", and in the two preliminary reports on our present work [Henry & Kon, 1937, 2; 1938] we fully endorsed this view. Indeed, the retention by young rats of over 98 % of the ingested Ca, not in a short metabolic period but in the course of 5 weeks during which they almost trebled their initial weights, can hardly be otherwise interpreted. For reasons more fully set out below we believe that it would be difficult to improve on the retentions observed in our present studies. Strictly speaking, therefore, the daily loss of some 0.3 mg. Ca may represent the unavoidable minimum. Two-thirds of it are due to urinary leakage of circulating Ca and the rest probably represents the Ca of digestive secretions which have not been reabsorbed, and the Ca of bacterial bodies. According to Sherman & Macleod [1925] the intestinal tract of the rat contains only negligible quantities of Ca and therefore the contribution of the shed epithelium is of no consequence in this respect.

We have just shown (p. 184) that under certain conditions the loss of Ca was primarily due to a relative shortage of P. The cause of the P loss is, however, not so easily explained. It is clear that it could not be due in the first instance to a shortage of Ca and addition of Ca to the diet did not improve the retention. It appears, therefore, that even under optimal conditions on the milk diets a definitely greater loss of P than of Ca is unavoidable. The difference lies mainly in the magnitude of the faecal excretions: 1.1 mg. daily, or 10.4 % of the intake, for P (Table XIIa, diet 93), as against 0.1 mg. daily, or 0.6 % of the intake, for Ca (Table XIIa, diet 91). The urinary losses on these diets were very similar: 0.15 mg. daily or 1.4 % of intake for P and 0.2 mg. or 1.3 % respectively for Ca. It is possible that a part of the faecal loss represents P that is not available. The diets contained some 57 % of maize starch with a P content of 0.016 % [Henry & Kon, 1937, 1]. If we assume that 50 % of this P is phytin-P—a generous estimate [cf. McCance & Widdowson, 1935]—and that 75 % of it is not available to the rat [Lowe & Steenbock, 1936], we arrive at an estimate of about 12 mg. faecal P due to unabsorbable phytin. This is, however, not enough to explain the difference between the faecal excretions of Ca and P, as at least 30 mg. of faecal P are not allowed for. It may be pointed out here that Brooke & Smith [1933] observed in rats on a ration very deficient in Ca and low in P daily faecal excretions of 0.3 mg. Ca and 1.3 mg. P and that Nicolaysen [1937] found in Ca and P starvation corresponding figures of 0.45 mg. Ca and 1.0 mg. P. Using a diet containing 0.15 % Ca and 0.4 % P (of which 0.21 % was derived from  $K_2HPO_4$ ) Rottensten [1938] noticed daily faecal excretions of 0.4 mg. Ca and 2.4 mg. P.

When allowance is made for the faecal P which may have been of phytin origin and, in the case of the diets containing  $CaHPO_4 \cdot 2H_2O$ , for the P from the unabsorbed Ca phosphate (cf. p. 185), there is evidence suggesting that the rest of the faecal P had been absorbed before it found its way into the gut. Thus, with diet 91, 99.4 % of the total Ca was absorbed (retained or excreted in the urine) which would leave practically the whole of the P in assimilable form. It seems therefore very probable that part of the faecal P was assimilated and then passed into the gut in the digestive juices and as shed epithelium. The findings of Johnson [1937] and Welch *et al.* [1936] on man, and Wright *et al.* [1938]

on the cat make it improbable that P is actively excreted in the large intestine. Intestinal epithelium doubtless contains much more P than Ca, but judging from the figures given by Johnson [1937] and Welch *et al.* [1936] for the P content of the material from isolated caeca, it is doubtful whether it makes an important contribution to faecal P. From the available recent analyses of digestive juices (cf. Baxter [1933] for mixed saliva, Gamble & McIver [1928], Ågren [1935], Ball [1930] and Walsh & Ivy [1928] for gastric and pancreatic juices; the latter, Drury [1924], Jones & Laing [1934] and Cheymol & Quinquaud [1937] for bile, and de Beer *et al.* [1935], Herrin [1935] and Wright *et al.* [1938] for *succus entericus*) it appears that they contain more Ca than P. Therefore, the mechanism for reabsorption of Ca must be much more efficient than that for P. It seems very probable that this is because a large part of the P present in the gut becomes fixed by the intestinal bacteria and is thus no longer available to the host. It has been shown by Osborne & Mendel [1914] that on low-residue diets like the diets used in the present study, bacterial bodies account for about 40 % of the dry weight of rat faeces. P is a more essential component of bacteria than Ca, and though both vary within very wide limits according to bacterial species and to the composition of the medium, authorities agree that bacterial ash contains more of the former than of the latter [Topley & Wilson, 1936; Buchanan & Fulmer, 1928]. Barber [1931], who analysed many species, gives for various coliform organisms values for P of about 1.5 % and for Ca around 0.2 % of the dry weight. It would be idle to speculate as to the exact P content of the bacterial flora of rats, nevertheless, the following calculation yields very plausible figures.

Table XIV. *Faecal excretion of P per g. food intake, P intake or per g. faeces*

	Total solid intake g.	P intake g.	Faecal P mg.	Wt. of faeces g.	Faecal P/g. total solid intake		Faecal P/g. P intake		Faecal P g. faeces	
					mg.	%*	g.	%*	mg.	%*
Raw and pas- teurized milk†	367.89	0.5132	81.25	8.69	0.2209	100	0.1583	100	9.35	100
Exp. 1										
Milk	129.03†	0.2038	23.20	2.49	0.1798	81	0.1138	72	9.32	100
Milk + Na <sub>2</sub> HPO <sub>4</sub>	132.12†	0.2415	25.30	2.62	0.1915	87	0.1048	66	9.66	103
Exp. 2										
Milk	148.19†	0.3420	30.20	3.80	0.2038	92	0.0833	53	7.95	85
Milk + Na <sub>2</sub> HPO <sub>4</sub>	146.67†	0.4468	35.60	3.87	0.2427	110	0.0797	50	9.20	98
Exp. 3										
Diet 89	341.75	0.4388	46.20	5.36	0.1352	61	0.1053	67	8.62	92
Diet 91	334.20	0.7910	49.90	5.01	0.1493	68	0.0631	40	9.96	107
Diet 93	301.76	0.3871	39.80	5.39	0.1319	60	0.1028	65	7.38	79

\* Taking the figures for raw and pasteurized milk as 100.

† Henry & Kon [1937, 1].

‡ On the assumption that the milk contained 12.5 % total solids.

Table IX shows that rats receiving diet 89 excreted on the average in the course of 5 weeks 5.36 g. of air-dry faeces. The faecal P output was 46.2 mg. (Table XIIa) or 8.61 mg. P per g. faeces (Table XIV). On the assumption that 1 g. faeces contained 400 mg. bacteria [Osborne & Mendel, 1914] this works out at 2 % P in bacterial bodies. A similar calculation for diet 91 gives 2.5 % P and for diet 93, 1.8 %; corresponding figures for Ca, omitting diet 93, are 0.22 % and 0.16 % respectively. The figures both for Ca and P are of the order quoted by Barber [1931] for various bacteria. If allowance were made for possible phytin-P not available to bacteria, the agreement with respect to P would be even better.



It seems therefore that as the faecal Ca on these diets can be entirely accounted for by Ca present in bacterial bodies the loss by this route could hardly be lowered. Moreover, the loss of 1.30 mg. Ca/g. faeces on raw milk diets (cf. Henry & Kon [1937, 1] and Exp. 1 of the present work) was greater than the figure of 0.77 mg. Ca/g. faeces obtained with the spray-dried milk diet in Exp. 3. It would appear therefore that drying, like pasteurization, has no untoward effect on the availability of milk-Ca for rats.

As a further argument in favour of the supposition that faecal P is mainly bacterial P, a comparison of all faecal P excretions on milk diets<sup>1</sup> shows that they agree much better when calculated per g. faeces than per unit of food intake or of P intake (Table XIV). A similar argument in favour of the bacterial origin of the faecal K has already been advanced by Peters [1933].

We are thus led to believe that there is no qualitative difference between the metabolism of Ca and that of P in the young growing rat. Neither Ca nor P can be adequately retained in the absence of sufficient quantities of P or Ca. When the available supply of the complementary element is adequate and that of the other is less than normal, only insignificant amounts of either Ca or P can leak out through the kidney.

The reabsorption of the quantities thrown into the gut with the digestive juices seems also to be equally efficient, the disparity in the amounts eventually lost not being due to any physiological or threshold differences but incidental to the symbiosis of host and intestinal flora. In neither case can we find any evidence of active excretion into the lower gut which, in the rat at any rate, does not in our opinion assume the functions of a vicarious kidney.

In our earlier experiments [Henry & Kon, 1937, 1] we found that when the supply of both Ca and P was inadequate, the requirements of the extra-skeletal structures for P were covered to a greater extent than those of the bones. Similar calculations applied to our present results yielded the values given in Table XV.

Table XV. *Exp. 3. Extent to which the requirements of the bones and extra-skeletal structures for P were satisfied by the rats receiving the experimental diets\**

Diet no.	% normal P (and Ca) in bones	% normal P in tissues
89	65.7	85.6
90	64.1	85.1
91	71.7	85.3
92	72.7	84.9
93	85.3	77.8
94	78.6	76.5
Liquid milk†	61.0	82.7

\* For method of calculation see Henry & Kon [1937, 1].

† Henry & Kon [1937, 1].

The results with diets 89 and 90, low in Ca and in P, agree very well with our previous findings. The addition of extra P (diets 91 and 92), and to a much greater extent the addition of extra Ca (diets 93 and 94), shift the P saturation from the soft tissues and blood to the bones which, when extra Ca is added,

<sup>1</sup> We have not carried out similar calculations for the diets containing Ca phosphate as they would be complicated by the presence in the gut of the unchanged compound (cf. pp. 177 and 185), especially as it was less well utilized in the preliminary experiments than in the final experiment. This can be easily seen by comparing the faecal excretions on milk and Ca phosphate diets in the three experiments.

appear to take precedence in their affinity for this element. It is possible that the inferior gain in weight of the rats on these diets may have had some effect on the result. It is of great interest, however, that Schneider & Steenbock [1938] have observed a similar effect in the presence of vitamin D on diets containing much more Ca than P.

## 2. *The availability of Ca and P from milk and from inorganic sources*

Experiments carried out on dogs some 20 years ago by Mendel and his collaborators [Givens & Mendel, 1917; McClugage & Mendel, 1918] showed that the Ca of milk was better utilized than that of Ca lactate or of  $\text{CaCO}_3$ . Previously, Aron & Frese [1908] had reported that the growing dog could obtain its Ca from Ca phosphate as efficiently as from milk. More recently Sherman & Campbell [1935; 1937] and Gaunt *et al.* [1938] showed that improvements in certain diets brought about by addition of milk could to a large extent be reproduced by the addition of  $\text{CaCO}_3$  or of Ca lactate and  $\text{Na}_2\text{HPO}_4$ . Fairbanks & Mitchell [1936] found that the Ca of a diet containing 0.18 % of the element, mostly in the form of dicalcium phosphate, was completely utilized by young growing rats. Stearns & Jeans [1934] found that children retained Ca and P approximately as well from  $\text{CaHPO}_4$  and  $\text{Ca}_3(\text{PO}_4)_2$  as from milk. Finally Coward *et al.* [1938] came to the conclusion that the Ca of pasteurized milk is no more easily available for the growing rat than the Ca of inorganic salts (Ca phosphate plus Ca lactate). It has already been pointed out by one of us [Kon, 1938] and also by Gaunt *et al.* [1938] that this conclusion was not justified by the available experimental data.

As far as they go these studies appear to indicate that if there is a difference between the availabilities of Ca from milk and from inorganic sources it cannot be very marked and our metabolic experiments confirm such a view. We find that though spray-dried milk is a better source of Ca than  $\text{CaHPO}_4$ , the difference between them under conditions of optimal retention is small. It should be pointed out that we used commercial Ca phosphate and that though the analysis of the salt tallied very well with the formula, it is possible that it was chemically not quite pure.

Another point to remember is that the degree of saturation of the body stores of Ca has a marked effect on the avidity with which the mineral is deposited [Fairbanks & Mitchell, 1936; Rottensten, 1938] and it is possible that the gap between milk and Ca phosphate as sources of Ca would be different at other levels of intake.

Ellis & Mitchell [1933] found that pasteurization lowered the availability of milk-Ca. We [Henry & Kon, 1937, 1] have been unable to confirm this finding. It is of interest that the figure obtained by us now for the availability of the Ca of a diet containing dried milk—98 %—is the same as that found by Ellis & Mitchell [1933] for a diet containing raw liquid milk. Since then Fairbanks & Mitchell [1937] have reported that Ca from spray-dried skim milk was some 7 % less available than the Ca from raw liquid skim milk, while Fairbanks & Mitchell [1938] obtained an average retention of Ca of only 85 % on a diet containing this type of dried milk. Already Ellis & Mitchell [1933] had expressed the view that the lowering of the availability of Ca in milk by pasteurization was due to a partial destruction of vitamin C. To this Fairbanks & Mitchell [1937] have added now what appears to be a striking confirmation. In preliminary experiments in which dried milk was fed to rats as a source of Ca, with and without the addition of 1 mg. daily of crystalline vitamin C, they found with a high

degree of probability that the administration of ascorbic acid improved the utilization of Ca. Our present experience is against such a view and the problem is now under investigation in our laboratory.

#### SUMMARY

1. The balance of Ca and of P derived either from milk or from  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  has been studied in three separate metabolic experiments on young male rats.

2. In all comparisons the Ca of milk was found to be more available than the Ca of  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ .

3. In the presence of extra P (as  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ) sub-optimal quantities of Ca were retained from spray-dried milk with an efficiency of 98.1% and from  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  with an efficiency of 96.5%. The difference between these two figures is significant.

4. Under such optimal conditions the daily loss of Ca is 0.3 mg. per rat. Of this one-third is lost in the faeces and two-thirds in the urine.

5. The retention of P is never so efficient, even in the presence of extra Ca (as  $\text{CaCO}_3$ ). The minimum daily loss observed was 1.3 mg. per rat. Of this 1.1 mg., or nearly 90%, was in the faeces.

6. It is suggested that this faecal P is mainly bacterial P and that the relatively inefficient retention of P at sub-optimal levels of intake is due to the fixation of some of this element by intestinal bacteria.

7. There is no evidence of active excretion of either Ca or P by the large intestine of the rat.

We are most grateful to Dr C. G. Anderson and to Prof. H. Raistrick for their helpful advice on the mineral constituents of bacteria and to Prof. Capstick for arranging the supply of the spray-dried milk.

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# XXII. THE EFFECT OF CONTINUED TREATMENT WITH ANTERIOR PITUITARY EXTRACTS ON MILK VOLUME AND MILK-FAT PRODUCTION IN THE LACTATING COW

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IN a previous paper [Folley & Young, 1938] it was shown that single injections into cows in declining lactation of crude preparations of prolactin and of thyrotropic hormone obtained from fresh ox anterior pituitary tissue, resulted in a substantial temporary increase in milk yield. On the other hand, a similar single injection of a prolactin preparation obtained from acetone-desiccated, commercial anterior lobe powder by a slight modification of the method of Bates & Riddle [1935], had no significant effect on the milk yield, although repeated daily injections of the same prolactin preparation caused a substantial increase in milk yield. In general it was found that in the cow the lactogenic action of the anterior lobe extracts used was paralleled by their glycotropic (anti-insulin) activity rather than by their prolactin (pigeon crop-gland stimulating) activity.

The present paper records the results of experiments in which the influence of repeated injections of prolactin and thyrotropic preparations with high glycotropic activity was investigated.

## METHODS

*Animals.* Thirteen dairy Shorthorn cows, belonging to the herd of the Reading University farm, were used in these experiments. Three groups, each of 4 animals, were selected so as to be as comparable as possible with regard to stage of lactation, time since mating if pregnant etc. A single cow was used in one experiment. The cows were allowed out to grass except when they came indoors for milking at approximately 8 a.m. and 4 p.m. each day. Their management was that usual for first-class dairy herds in this country.

*Milk analyses.* In addition to daily milk yield, percentages of fat and non-fatty solids in group-composite samples were determined twice daily throughout the experiment on the 3 main groups. The content of lactose, total chlorides and phosphatase was determined at intervals. The methods used for these determinations were those previously described [Folley & Young, 1938].

*Anterior lobe extracts.* Preparations "Prolactin-C" and "Thyrotropic-C" [Folley & Young, 1938] were used in this investigation. The method of preparation of these extracts and their general properties have already been described [Young, 1938, 1; Folley & Young, 1938]. In brief, the method of preparation consisted in the extraction of absolutely fresh ox anterior pituitary lobes with saline at pH 8 in the cold, followed by separation at 0° into the fractions soluble at pH 5.5 and those insoluble at this pH. The former constituted "Thyrotropic-C" and the latter "Prolactin-C".

*Injection of animals.* After a suitable control period, each of the 4 cows in Group A was given, on alternate days, an injection of 30 ml. of "Prolactin-C" equivalent to 10 g. of fresh anterior lobe tissue. Each cow in Group B (4 animals) received similar injections of "Thyrotropic-C". Group C served as a control group and each of these 4 animals was injected on alternate days with 30 ml. of a saline suspension of fresh ox liver equivalent to 10 g. of fresh tissue. These injections provided an adequate control for the possible influence on milk secretion of the introduction of non-specific protein into the circulation of lactating cows.

Repeated injections of "Prolactin-C", but not of the other tissue extracts used in the present investigation, produced local reactions of the type produced by injections of a crude saline extract of ox anterior lobe tissue [Folley & Young, 1938]. It was found that centrifuging the "Prolactin-C" preparation at pH 8 threw down material that was normally in suspension [cf. Young, 1938, 1] leaving a clear fluid. One cow received injections of 30 ml. of this fluid (equivalent to 10 g. of fresh tissue) on alternate days for 25 days and showed no local reactions.

## RESULTS

*Milk yield.* The curves for aggregate milk yield in the 3 main groups are shown in Fig. 1. The daily yield of the cows receiving 5 injections of Prolactin-C was very substantially increased during the period of injections. Although the

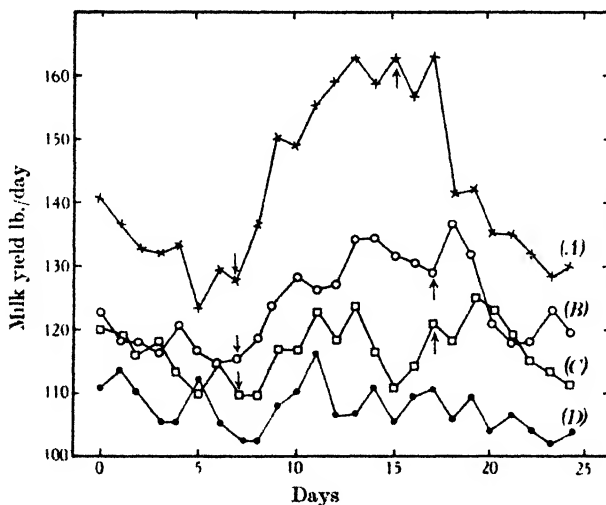


Fig. 1. Aggregate milk yields of groups of 4 cows receiving injections of pituitary extracts on alternate days. The amount of extract injected on each occasion was equivalent to 10 g. of fresh ox anterior lobe. ↓ signifies the first injection. ↑ signifies the last injection. Curve A: animals receiving Prolactin-C. Curve B: animals receiving Thyrotropic-C. Curve C: control animals receiving liver extract. Curve D: uninjected control animals.

milk yield did not continue to increase over the whole period of the injections, there was no sign of a decline from the highest level reached, until after the injections were discontinued.

Injections of Thyrotropic-C also increased the aggregate milk yield, although to a much less extent than those of Prolactin-C (Fig. 1), and again

there was no definite diminution of the increased milk yield until the injections had ceased.

In the group receiving liver extract a slight increase in milk yield occurred immediately after the initial injection. Unfortunately, on the day on which injections began it was found necessary to turn all the cows used in this experiment into a new pasture. In a normal year when grass is plentiful this would have made very little difference to the milk yield, but this experiment was carried out in the summer of 1938 when grass was very scarce owing to drought. Under these conditions a change to a new pasture might be expected to exert a stimulating effect on milk yield. That this was so is shown by the aggregate milk yield curve for the same period for a group of 4 untreated cows of the same herd (Fig. 1). The slight increase in milk yield which occurred following the injections of the liver extract cannot therefore be ascribed to a lactogenic action of this extract.

The milk yield curve for the cow which received injections of the centrifuged Prolactin-C is shown in Fig. 2. In this experiment a total of 12 injections was

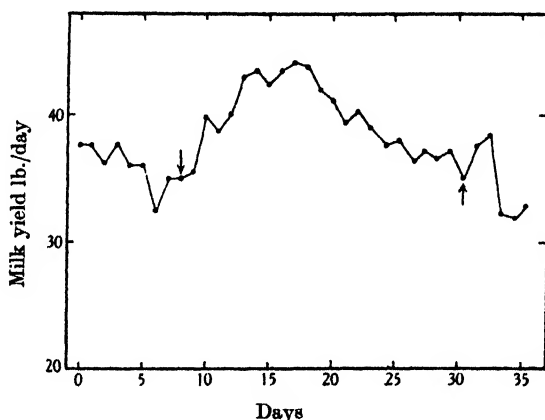


Fig. 2. Milk yield of a cow receiving injections of centrifuged Prolactin-C on alternate days. The amount of extract injected on each occasion was equivalent to 10 g. of fresh ox anterior lobe. ↓ signifies the first injection. ↑ signifies the last injection.

given, material being injected on alternate days. The milk yield rose to a maximum on the 10th day of the experiment, after which it steadily decreased, despite continued treatment.

**Milk-fat percentage.** The percentage of fat in the milk of the cows in Groups B and C (receiving injections of Thyrotropic-C and liver extract respectively) was unchanged during the experimental period (Fig. 3). The milk-fat percentage in the animals in Group A (receiving injections of Prolactin-C) was, on the other hand, very substantially increased as the result of the injections. As both the milk yield and the percentage of milk-fat were substantially raised, the total amount of fat secreted in the milk of these animals was very greatly increased as the result of treatment (Fig. 4). The total daily production of milk-fat by the cows in this group averaged 4.48 lb. for the 7 days before the injections were begun, and 6.63 lb. for the 10 days covering the period of the injections. Thus the daily production of milk-fat was increased by 48 %, on the average, following the institution of daily injections of Prolactin C.

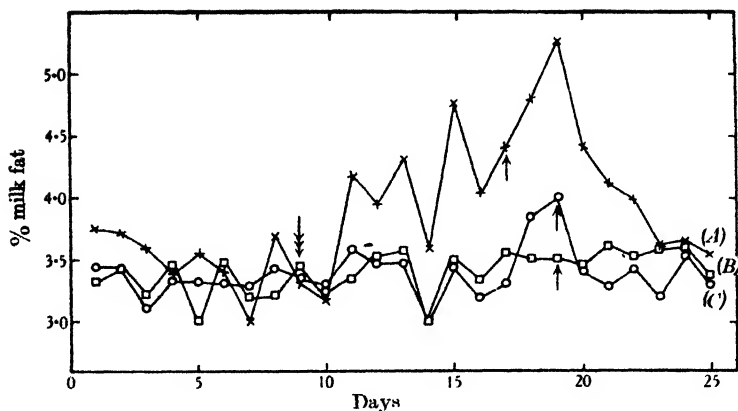


Fig. 3. Percentage milk-fat in pooled samples of the milk from groups of 4 cows receiving injections of pituitary extracts on alternate days. The amount of extract injected on each occasion was equivalent to 10 g. of fresh ox anterior lobe. ↓ signifies the first injection. ↑ signifies the last injection. Curve A: animals receiving Prolactin-C. Curve B: animals receiving Thyrotropic-C. Curve C: animals receiving liver extract.

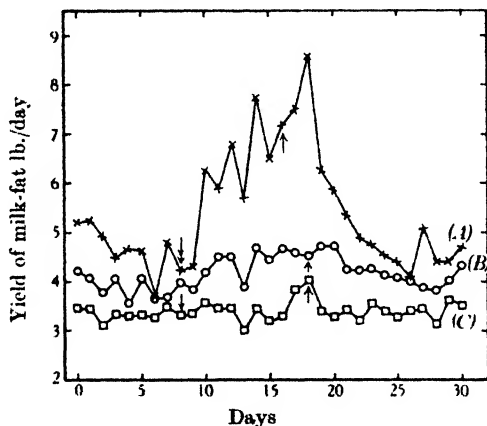


Fig. 4. Aggregate yields of milk-fat from groups of 4 cows receiving injections of pituitary extracts on alternate days. ↓ signifies the first injection. ↑ signifies the last injection. Curve A: animals receiving Prolactin-C. Curve B: animals receiving Thyrotropic-C. Curve C: animals receiving liver extract.

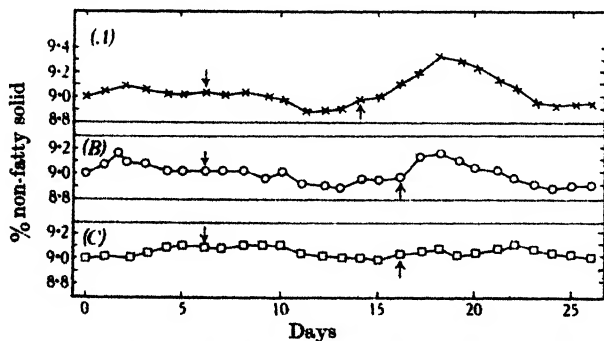


Fig. 5. Percentage non-fatty solid in pooled samples of the milk from groups of 4 cows receiving injections of pituitary extracts on alternate days. ↓ signifies the first injection. ↑ signifies the last injection. Curve A: animals receiving Prolactin-C. Curve B: animals receiving Thyrotropic-C. Curve C: animals receiving liver extract.



*Non-fatty solid content of milk.* The milk from the cows receiving injections of anterior lobe extract showed no clear indication of a significant change in non-fatty solid content (Fig. 5), there being a slight but probably insignificant fall. Lactose, chloride and phosphatase contents showed no regular alterations during treatment.

#### DISCUSSION

These experiments show that the rate of milk secretion of cows in declining lactation can be increased by repeated injections of preparations of prolactin and of the thyrotropic substance, both possessing marked glycotropic activity, to a level much higher than was attained with single injections of these preparations. The single experiment in which 12 successive injections of prolactin were given showed that with prolonged treatment the milk yield reaches a maximum value and then declines, despite repeated injections. It does not seem probable that this decline was due to the production of an "antihormone" in the injected animal, as daily injections of ox-prolactin for 12-15 weeks were required to induce the appearance of substantial "anti-prolactin" activity in rabbits [Young, 1938, 2].

The great increase in milk-fat content as the result of treatment with prolactin was unexpected in view of the simultaneous increase in milk yield which occurred. The total production of milk-fat by each cow was increased by over 0.5 lb. a day as the result of this treatment. This increase is particularly interesting when it is remembered that the daily injection of a prolactin preparation relatively poor in glycotropic activity resulted in no significant increase in milk-fat content, although the milk yield was substantially increased thereby [Folley & Young, 1938]. There is no evidence whether or not the glycotropic substance influences fat metabolism, but it should be recalled that Marks & Young [1938] found that treatment of young rabbits with prolactin and thyrotropic preparations, both high in glycotropic activity, resulted in a substantial increase in liver fat. The glycogen content of the livers of these animals was also abnormally high. Similar results have been obtained with adult rabbits and with other species of animals (unpublished observations). It is not known at the present time how many active substances are present in anterior pituitary extracts of the type used in the present investigations. Until further fractionation of the active substances has been effected one can only attempt to correlate the observed physiological activities of the different fractions used. Table I summarizes the different physiological activities of the prolactin and thyrotropic preparations used in the present investigation. There is no clear indication of any correlation of the activity in increasing milk-fat content with any of the other activities investigated.

Table I. *Summary of physiological activities of different extracts used*

Physiological activity	Reference	Pro-lactin-C	Thyro-tropic-C
Glycotropic (anti-insulin) activity	Young [1938, 1]; Marks & Young [1938]	+++	++
Increased liver fat content of fasting rabbits	Marks & Young [1938]; and unpublished data	+++	++
Increased liver glycogen content in fasting rabbits	Young [1937]; Marks & Young [1938]	++	+++
Milk yield increase in cows in declining lactation	Folley & Young [1938]; and present communication	+++	++
Increase in milk-fat content in cows in declining lactation (repeated injection of extract)	Present communication	+++	0

. The injection of thyroxine into cows in declining lactation results in an immediate increase in milk yield, together with a delayed increase in the fat content of the milk [Folley & White, 1936]. The above results cannot be due to the thyrotropic activity of the pituitary extracts used, as the prolactin-C was most potent in influencing milk yield and fat content and this preparation has only a very slight thyrotropic activity [cf. Young, 1938, 1].

The results recorded in the present paper emphasize the undesirability of attempting to ascribe to any single substance of the pituitary gland stimulation of all the processes concerned with the secretion of milk. It is clear that in cows in declining lactation, neither the increase in milk yield nor the increase in milk-fat content, both of which are found to follow the repeated injection of a suitable pituitary extract, are related to the activity of the extract in causing growth of the pigeon crop-gland. The determination of how many different factors are involved in the control and stimulation of the complex processes of milk formation must await further fractionation of the extracts used.

#### SUMMARY

1. Repeated injections of thyrotropic and prolactin preparations into cows in declining lactation resulted in a pronounced increase in milk yield, the increase being more marked with the prolactin preparation.

2. The increase in milk yield following injections of the prolactin preparation was accompanied by a substantial increase in milk-fat content. The average daily production of milk-fat was increased by nearly 50 % during the period of 5 successive injections of prolactin.

3. Under prolonged treatment with prolactin the milk yield did not continue to increase, but reached a steady level and then declined, despite continued treatment.

4. The increase in milk yield and milk-fat content of cows in declining lactation, following the injection of pituitary extracts, cannot be ascribed to the action of a single substance.

We are indebted to Miss H. M. Scott Watson for valuable help with the calculation of results, and to Mr S. Watson for technical assistance. The co-operation of Prof. R. Rac and Mr K. W. D. Campbell of the University of Reading Farm, is gratefully acknowledged.

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## XXIII. VITAMINS OF ELEPHANT'S MILK

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FOLLOWING the birth of an elephant at the Warsaw Zoological Garden in June 1937 (the twelfth recorded case in Europe), a study of the chemical composition of elephant's milk was undertaken by Dr J. Królikowski of the Department of Genetics and Animal Dietetics of the School of Agriculture, Warsaw. The present paper describes the results of a comparison of the vitamin content of the milk, supplied to us by Dr Królikowski, with that of cow's milk. The samples of milk were taken periodically over the term of a year, commencing 8 weeks after parturition, one teat being milked while the calf was sucking another: the milk was delivered at the laboratory within an hour of milking. The staple food of the mother over the period consisted of grass, hay, wheat-bran, oil-cake, carrots and fodder beets. Prior to parturition, the fodder was supplemented with milk, and immediately after calving steamed linseed and wheat-bran were also given.

In view of the limited amount of milk available, the various vitamins were not determined simultaneously, but successively. This somewhat detracts from the value of the results obtained, since the relative contents of the individual vitamins may vary according to the season of the year, duration of lactation, nature of fodder and other variable factors. Raw cow's milk supplied by the Piaseczno Dairy was taken for comparison; the cows were fed on hay, grass, wheat-bran, oil-cake, soya bean, fodder beets and swedes. Studies were made of vitamins A, B<sub>1</sub>, the B<sub>2</sub> group, C and D.

### *Experimental*

*Vitamin A.* This was assayed by the curative method on rats given Mellanby's vitamin A-deficient diet [Green & Mellanby, 1928]. The increase in weight during a 4-week period of administration of milk was compared with that produced by the addition of 2-3 I.U. daily of vitamin A (International standard). The experiments were conducted in March and April (11th and 12th months of lactation). The results are given in Table I.

Table I. *Vitamin A*

Source of vitamin	Daily dose	No. of rats in group	Duration of experiment days	Mean wt. increase g.
International standard	2 I.U.	3	28	24
	3 „	3	28	37
Cow's milk	2 ml.	6	29	30
Elephant's milk	2 „	9	28	3
	2.5 ml.	4	28	5

The symptoms of xerophthalmia disappeared from rats given the International standard or 2 ml. of cow's milk daily, but not from those receiving elephant's milk. The latter animals did not increase in weight and at necropsy

exhibited the characteristic suppurative foci of avitaminosis-A. It may be concluded that the milk contained less than 1 I.U. of vitamin A per ml.

*Vitamin B<sub>1</sub>*. This was assayed by the curative method on rats aged 3-4 weeks. Table II gives the average increase in weight of groups of rats maintained for 3 weeks on Evans' fat-free vitamin B<sub>1</sub>-deficient diet [Evans & Lepkovsky, 1929], supplemented with varying amounts of milk, or with the International standard vitamin B<sub>1</sub> preparation (1 unit daily). The assays were carried out from September to November (5th to 7th months of lactation).

Table II. *Vitamin B<sub>1</sub>*

Source of vitamin	Daily dose	No. of rats in group	Duration of experiment days	Mean weekly wt. increase g.
International standard	10 mg. (= 1 I.U.)	6	21	10.0
Cow's milk	8 ml.	5	21	4.0
	10 "	4	21	5.0
Elephant's milk	3 "	5	21	7.5
	5 "	5	21	14.0
	8 "	5	21	14.5

It follows from the results obtained that elephant's milk contains  $\approx 25$  I.U. of vitamin B<sub>1</sub> per 100 ml., compared with  $< 10$  I.U. in cow's milk.

*Vitamin B<sub>2</sub> complex*. This complex was assayed on rats weighing 50-60 g. Their mean weight increases during 4 weeks on a diet deficient in B<sub>2</sub>-vitamins [Sherman & Spohn, 1923], supplemented with 2 I.U. daily of vitamin B<sub>1</sub> and with 4 ml. daily of cow's or elephant's milk, are given in Table III. The assays were made from July to September (3rd to 5th month of lactation).

Table III. *Vitamin B<sub>2</sub> complex*

Source of vitamin	Daily dose	No. of rats in group	Duration of experiment days	Mean weekly wt. increase g.
Elephant's milk	4 ml.	5	28	10.7
Cow's milk	4 "	5	28	13.0

It may be concluded that the amounts of vitamin B<sub>2</sub> complex are approximately the same in cow's and elephant's milk.

*Vitamin C*. This was determined chemically according to the modification by Birch *et al.* [1933] of the method of Tillmans [1932]. Kon & Watson [1936] have shown that ascorbic acid is readily converted in the presence of light into dehydroascorbic acid, which does not react with Tillmans' reagent and which is reduced to ascorbic acid by H<sub>2</sub>S. Reducing substances other than ascorbic acid were removed by the method of Emmerie and Van Eekelen [1934].

The ascorbic acid content of elephant's milk, tested in February (10th month of lactation), was 8.80 mg. per 100 ml. for skimmed, and 7.72 mg. for full cream. milk; the corresponding values for cow's milk were 2.18 and 2.12 mg. per 100 ml.

*Vitamin D*. This vitamin was assayed by the preventive method on rats receiving the rachitogenic diet No. 2965 of Steenbock & Black [1925], supplemented with 10, 12 or 15 ml. of elephant's milk daily, or with 1 I.U. of calciferol (international standard). The rats were sacrificed on the 30th day and epiphyseal ossification was examined by the line test and by means of X-ray photographs. The rats receiving 10 and 12 ml. of milk had well-marked rickets, not differing in severity from vitamin D-deficient controls. Those given 15 ml. of milk daily

suffered from mild rickets, whilst those receiving 1 unit of international standard preparation showed normal ossification. Comparison with cow's milk was not performed in this case, but the author has found (unpublished) that 10 ml. of cow's milk contain about 1 I.U. of vitamin D. The assays were performed in November and December (7th and 8th months of lactation).

#### SUMMARY

1. Elephant's milk has a lower content of fat-soluble vitamins (A and D) than has cow's milk.
2. Its vitamin B<sub>1</sub> content exceeds that of cow's milk, being 25 I.U. per 100 ml., as compared with 10 I.U. for the latter.
3. Elephant's and cow's milks have approximately the same content of vitamin B<sub>2</sub> complex.
4. Elephant's milk contains 7.72 mg. per 100 ml. of ascorbic acid, as compared with 2.12 mg. in cow's milk.

The author wishes to express her gratitude to Dr J. Zabiński, director of the Warsaw Zoological Gardens, for his co-operation, and to Dr R. Truszkowski for his helpful interest.

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# XXIV. STUDIES IN THE STABILITY ON VITAMINS A AND D

## II. ACTION ON FATTY PEROXIDES ON VITAMIN A

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THE present widespread use of vitamin A in medicine calls for information as to its stability, particularly when dissolved in oils. Several workers [Huston *et al.* 1928; Evers, 1929; Norris & Church, 1930; Holmes *et al.* 1936] have studied the stability under different conditions of storage of vitamin A in fish liver oils and of vitamin A concentrates dissolved in vegetable oils. Wide variations in the rate of destruction of vitamin A have been recorded: Jones [1928] found a reasonable amount of vitamin A present in a malt extract and cod liver oil preparation 23 years old; at the other extreme Marcus [1931] found vitamin A to be largely destroyed in 3 hr. when dispersed on decolorizing charcoal. Other workers [Wokes & Willimott, 1927; MacWalter, 1934] have studied the rate of destruction of vitamin A in cod liver oil aerated at various temperatures. Dann [1932] investigated the rate of destruction of vitamin A in a cod liver oil concentrate dissolved in a range of solvents, on aeration at 98°, and found surprising variations from complete stability in certain alcohols and esters to rapid destruction in fatty acids. Fridericia [1924] and Powick [1925] found that admixture with rancid lard slowly destroyed vitamin A in cod liver oil, and suggested that the effect might be due to organic peroxides. Rosenheim & Webster [1926] found peroxides present in cod liver oil in which the vitamin A had disappeared on exposure to air and light. Wokes & Willimot [1927] suggested that the destruction of vitamin A caused by aerating cod liver oil was due to the formation of volatile organic peroxides, while Whipple [1936] attempted to correlate the peroxide value with the destruction of vitamin A in cod liver oil as rancidity developed. On the other hand, Griffiths *et al.* [1933] found that vitamin A was stable for at least 4 months in cod liver oil emulsions prepared with gum acacia containing peroxidase. Cady & Luck [1930] found that emulsification of cod liver oil with  $H_2O_2$  only slowly destroyed the vitamin A present, and Dann [1932] found that vitamin A was stable in a cold alcoholic solution of  $H_2O_2$  for 24 hr.

We have made a number of experiments on the stability of vitamin A (in the form of concentrates) dissolved at various strengths in various vegetable oils, with and without the addition of 0.05% quinol as antioxidant. In all instances the vitamin A content (measured by the absorption at 328 m $\mu$ ) diminished, sometimes to vanishing point, during the course of 2 years' storage, but the rates of destruction were extremely erratic, even to the extent that the same solution in different bottles deteriorated at different rates. In general, there was a tendency for the more dilute solutions (1200 and 12,000 I.U./g.) to deteriorate more rapidly than the stronger ones (60,000 I.U./g.). In some instances quinol

had a slight retarding effect on the destruction but in others it appeared inactive. This is contrary to the findings of Norris & Church [1930] and of various other workers who have used quinol to protect the vitamin A in solutions for animal feeding, but it must be noted that the solutions we tested were very much more concentrated. Many of the solutions were rancid at the end of the test, but vitamin A was still present in some of these rancid solutions, whereas little or none was present in some of the solutions which neither tasted nor smelt rancid. Nevertheless, it seemed that the most probable explanation of the erratic nature of these findings was that fatty peroxides were the agents immediately destructive of the vitamin A, since it is well known that the rate of development of peroxides in oils is affected by conditions of storage, the extent of exposure to air, and particularly by the amount of natural antioxidants present.

A number of samples of various vitamin A concentrates at a uniform strength of 12,000 I.U./g. in arachis oil stored for many months in partly filled bottles were available. These were examined both for the content of vitamin A and of peroxides (by a slight modification of Lea's method [1929]). It was found that always when the peroxide value exceeded 5, at least 70 % of the vitamin A had disappeared. All samples in which little destruction had occurred showed very low peroxide values, but nevertheless there were several samples with low peroxide values in which considerable destruction of vitamin A had occurred. It is, of course, possible that these samples had at some time contained more peroxides, which had been used up in destroying the vitamin A, or in oxidizing constituents of the oil.

A number of experiments were then made in order to test more rigorously the theory that fatty peroxides slowly oxidize vitamin A. In the first series, a number of samples of vitamin A in the form of rich fish liver oils and concentrates of different types were dissolved to a strength of 12,000 I.U./g. in a rancid olive oil having a peroxide value of 260.<sup>1</sup> The solutions were immediately transferred to a series of glass tubes, which were evacuated, filled with N<sub>2</sub> and sealed. The tubes were then heated in a boiling water bath, and at intervals a tube was removed and the vitamin A determined by measuring the absorption at 328 m $\mu$ . It was observed that a progressive bleaching of the solutions occurred, while the absorption at 328 m $\mu$  steadily diminished with time of heating. Further, the trough of the absorption curve on the short-wave side of 328 m $\mu$  became filled up as the reaction proceeded, until the later samples showed only general absorption. In every instance the actual absorption at 328 m $\mu$  was measured, irrespective of whether or not the curve showed a maximum at this wave-length. All values were corrected for the slight general absorption of the solvent oil at 328 m $\mu$ . Every experiment was done at least in duplicate. The results are shown graphically in Fig. 1. It will be observed that the curves fall into 2 groups. The curves corresponding with the more rapid destruction of vitamin A cover 3 samples of vitamin A concentrate, 2 from fish liver oil and 1 from mammalian liver. The curves corresponding with slower destruction of vitamin A cover two fish liver oils (one of them of exceptionally high potency). It has previously been shown [Bacharach & Smith, 1928] that vitamin A exists in fish liver oil in a state different from that in which it is present in concentrates, and it was suggested that vitamin A is an alcohol, which in fish liver oils is esterified with fatty acids. It is interesting to note that this difference is reflected in a greater resistance of the esterified vitamin A to oxidation by fatty peroxides. The matter was further tested by esterifying a vitamin A concentrate with stearic acid, and

<sup>1</sup> As defined by Lea; each g. of oil contained peroxide oxygen equivalent to 260 ml. of N/500 thiosulphate.

measuring the rate of destruction of the vitamin A by the rancid olive oil. The data are included in Fig. 1, and it will be seen that the points lie close to those for the fish liver oils.

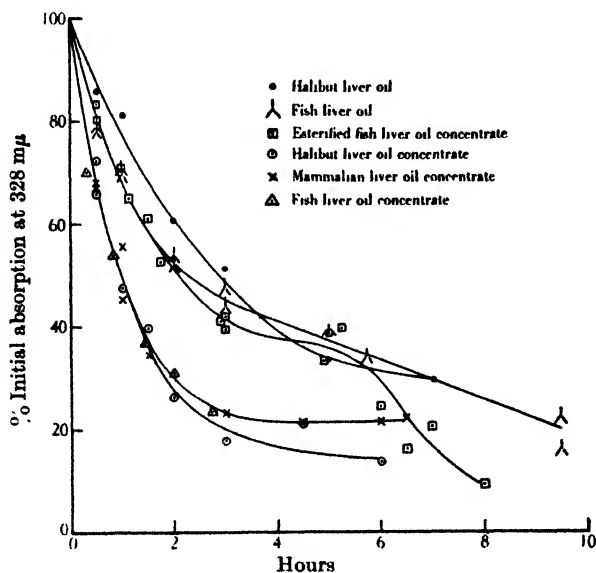


Fig. 1.

Fig. 1 shows clearly that the absorption at  $328\text{ m}\mu$  tends not towards zero, but towards a value of some 10–20% of the initial absorption. The probable explanation of this phenomenon is that the primary reaction concerns only one of the double bonds in the vitamin A molecule, and that the primary oxidation product has an absorption band at a shorter wave-length than that due to vitamin A, but broad enough to show significant absorption at  $328\text{ m}\mu$ . Some confirmation of this was afforded by the  $\text{SbCl}_3$  colour test. A solution in rancid oil after  $8\frac{1}{2}$  hr. heating showed 11% of the initial absorption at  $328\text{ m}\mu$ ; the colour test indicated only 7% of the initial vitamin A and, in addition, the test was abnormal; the solution became purple on adding the reagent, and the colour rapidly changed to blue, the reverse of the normal change. It is quite conceivable that some such colour reaction would be given by a primary oxidation product of vitamin A. There is some indication from the shape of the curves, in particular that for the artificially esterified vitamin A, that this primary oxidation product is itself slowly attacked by peroxide oxygen, with the production of a substance showing less absorption at  $328\text{ m}\mu$ . It is certainly possible for vitamin A to be oxidized to a substance almost completely transparent at  $328\text{ m}\mu$ , for we have had oil solutions decrease in absorption with development of rancidity to 1.5% of the initial value, and this residual absorption was probably due to the glycerides.

In order to prove more definitely that the fatty peroxides themselves and not some other constituent of rancid oil were the actual destructive agents, a series of oils were prepared having different peroxide values. Olive oil was chosen as a typical vegetable oil and coconut olein as an oil mainly containing shorter carbon chains. Samples of these oils were exposed to the air in a warm place, while others were heated at  $100^\circ$  and others irradiated with a quartz



mercury vapour lamp. A sample of vitamin A concentrate was dissolved in each of the oils to a strength of 9600 I.U./g. and the samples were sealed in N<sub>2</sub> as before. They were all heated for the same times, namely, 2 and 4½ hr. at 100° and 45 hr. at 50°. The final vitamin A contents are shown in Table I, where the

Table I. *Fish liver oil concentrate dissolved in oils to 9600 I.U./g.*

Oil	Peroxide value ml. N/500 Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> per g.	% original $E_{1\text{cm}}^{1\%}$ 328 mμ after		
		2 hr. at 100°	4½ hr. at 100°	45 hr. at 50°
Coconut olein V	0.5	96	—	95
Coconut olein VI	4.8	90	82	(87)
Olive oil I	10.5	83	81	94
Coconut olein VII	15	84	59	—
Olive oil II	18	79	65	96
Coconut olein VIII	36	74	57	87
Coconut olein IX	50	57	45	73
Olive oil III	64	57	39	73
Olive oil IV	125	41	22	63
Undiluted concentrate		94	92	96

oils are arranged in order of increasing peroxide value, irrespective of their nature (olive oil or coconut olein) or the method by which peroxide formation was induced. It will be observed that with very few exceptions this order is also the order of decreasing vitamin A content in the heated samples. The experiment was repeated with a sample of artificially esterified concentrate dissolved in some of these oils at a strength of 12,000 I.U./g. (Table II). No measurable destruction

Table II. *Esterified fish liver oil concentrate dissolved in oils to 12,000 I.U./g.*

Oil	Peroxide value ml. N/500 Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> per g.	% original $E_{1\text{cm}}^{1\%}$ 328 mμ after		
		2 hr. at 100°	4½ hr. at 100°	100 hr. at 45°
Coconut olein V	0.5	100	100	—
Coconut olein VI	4.8	—	100	98
Olive oil I	10.5	100	100	100
Coconut olein VIII	36	91	88	88
Olive oil III	64	83	79	97
Olive oil IV	125	75	70	84

occurred, except with oils having a peroxide value of 36 or higher, but again (with one exception) increasing peroxide value went parallel with decreasing vitamin A; destruction was again much slower than with the unesterified concentrate.

No attempt was made to confirm these findings by biological assays, mainly because it was to be expected that the fatty peroxides would cause further destruction of vitamin A during the course of the assay or during any attempt to remove the peroxides, e.g. by saponification.

Some preliminary experiments along similar lines were done with solutions of calciferol in rancid olive oil, but it was impossible to assay the vitamin D accurately. Biological assays were contra-indicated, for the reason mentioned in connexion with vitamin A. There were indications that the oxidation products of vitamin D interfere with its direct spectrophotometric determination and even more with its determination by the Brockmann method with SbCl<sub>3</sub>. Nevertheless, these experiments showed that calciferol is far more resistant than is vitamin A to destruction by fatty peroxides. Conditions that caused almost complete destruction of vitamin A caused barely 10% destruction of vitamin D.

## DISCUSSION

In most of these experiments the concentration of peroxide oxygen was vastly in excess of that of the vitamin A, so that the reaction would be expected to follow a unimolecular, i.e. logarithmic law. When appropriate allowances were made for the absorption at 328  $m\mu$  of the first oxidation product postulated, the experimental results were found to follow approximately the logarithmic law.

In the second series it would be expected that the velocity of reaction would be proportional to the peroxide value. This was found to be the case within the limits of error of the spectroscopic determinations, except that the vitamin A in the solutions of low peroxide value was destroyed more rapidly than was to be expected. The indication here is that some factor other than peroxide oxygen caused very slow destruction of vitamin A. The fact that slow destruction of the undiluted vitamin A concentrate occurred when it was heated in  $N_2$ -filled tubes alongside the oil solutions suggests that this factor is simple heat. Edisbury *et al.* [1932] and Robinson [1938] have shown that heat can cause destruction of vitamin A in absence of  $O_2$  or any oxidizing agent.

## CONCLUSIONS

These experiments appear to afford clear evidence that fatty peroxides can cause destruction of vitamin A rapidly at 100° and more slowly at 50° and at room temperature. A practical consideration which follows is that the peroxide value of oil solutions of vitamin A needs to be considered, not merely as an index of potential rancidity, but as an index of the stability of the vitamin A. Moreover, a peroxide value not high enough to cause concern as to the stability of the oil itself may well be sufficient to cause slight destruction of dissolved vitamin A. The evidence that is here presented confirms that given in the previous paper of this series [Robinson, 1938] to the effect that oxidation products of vitamin A may show considerable absorption at 328  $m\mu$ . It follows that with vitamin A preparations that have suffered oxidation, a considerable portion of the absorption at 328  $m\mu$  may be due to substances other than vitamin A. Thus the present work affords further evidence that a spectrophotometric assay must be regarded with some suspicion when the band at 328  $m\mu$  shows poor persistence.

Fatty acid esters of vitamin A are more stable than the free alcohol towards fatty peroxides. This might be due either to the participation in the reaction of the hydroxyl group present in the free alcohol or, perhaps more likely, to activation by the hydroxyl group of the conjugated double bond system, rendering it more susceptible to attack by peroxide oxygen.

## SUMMARY

Vitamin A held in oil solution at room temperature disappeared at widely varying rates, but in general, destruction was greatest in solutions that developed a high peroxide value. Liver oils and concentrates were dissolved in rancid olive oil of peroxide value 260 and heated to 100° in  $N_2$ -filled sealed tubes. Progressive destruction of vitamin A occurred, more rapidly with the concentrates (of fish and mammalian origin) than with the fish liver oils (in which vitamin A occurs as an ester) or a re-esterified concentrate.

Oxidation of the vitamin A gave rise to a product with unselective absorption at 328  $m\mu$ , so that when oxidation has occurred the total absorption at 328  $m\mu$  indicates values in excess of the true vitamin A content.

Two concentrates were dissolved in samples of olive oil and coconut olein showing a wide range of peroxide values and the solutions sealed into tubes. After heating of the tubes under identical conditions, the extent to which the vitamin A had been destroyed was approximately proportional to the peroxide value and independent of the nature of the oil.

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# XXV. STUDIES IN THE STABILITY OF VITAMINS A AND D

## III. THE EFFECT OF LIGHT ON VITAMIN A

BY ERNEST LESTER SMITH, FRANK ARNOLD ROBINSON,  
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*(Received 21 December 1938)*

It is well known that the vitamin A in an oil or concentrate gradually disappears on aeration or storage. It has also been recorded that vitamin A is destroyed by irradiation [Chevallier & Choron, 1934; Chevallier, 1935; 1938; De, 1935; Morton & Heilbron, 1928; Morton *et al.* 1931; Zilva, 1919]. On the other hand, Boyle [1934] obtained a large increase in the value of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  and in the Carr-Price value of a turbot-liver concentrate when this was irradiated for 20 min. Such a large increase has not been observed by other workers, though small increases in the value of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  have been found to occur during storage of oils and concentrates for a short time, both by ourselves and by other workers [Gillam & Morton, 1931; Griffiths *et al.* 1933; Heilbron *et al.* 1931; MacWalter, 1934; Garratt, 1938]. The increase was in every instance slight and was always followed by a steady decrease. Chevallier & Choron [1934] claim that on irradiation of vitamin A a substance  $A^1$  is first formed which is sparingly soluble in ethyl alcohol, but readily soluble in hexane, and has an absorption band with a maximum at 312.5  $m\mu$ ; further irradiation results in the formation of  $\beta$ -ionone.

We have ourselves confirmed the findings of the majority of workers that a decrease in the value of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  occurs when solutions of oils or concentrates containing vitamin A are irradiated, but we have encountered a new phenomenon, a brief report of which has already been published [Smith *et al.* 1938]. When a solution of vitamin A was irradiated and  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  was measured immediately, and again after standing in the dark for a few hr., it was found that the latter value was greater than the former. Furthermore, after a brief irradiation, causing a relatively small decrease in  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$ , the value obtained after standing in the dark was found to be about the same as that of the unirradiated solution. More prolonged exposure to the irradiation was followed by a smaller degree of recovery on standing in the dark. As might be expected, the effect of irradiation was most marked with intense ultraviolet light, e.g. from a mercury vapour lamp, but a decrease in the value of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  was also observed after several hours' exposure to daylight. In order to bring about an increase of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  after intense irradiation it was not necessary for the irradiated solution to be kept in complete darkness; storage in daylight resulted in some recovery. Thus exposure to daylight brought about a decrease in  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  if the solution had previously been kept in the dark, but it resulted in an increase in  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  if the solution had previously been exposed to intense radiation.

## EXPERIMENTAL

All absorption spectra were measured by means of a Hilger E2 Medium Quartz Spectrograph  $F_D$  60 cm. with an H237 Spekker Photometer using tungsten-steel electrodes and a high-voltage spark. A range of exposures from

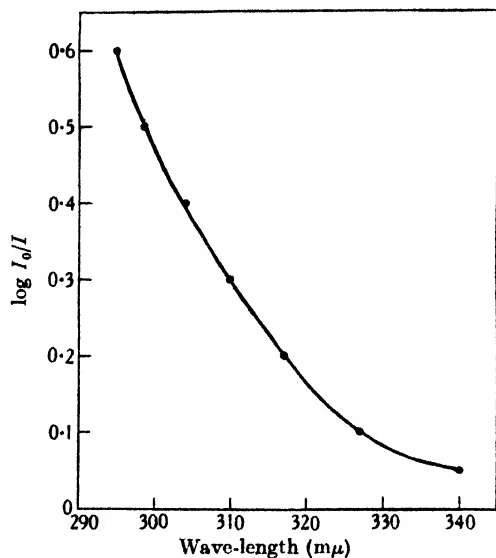


Fig. 1. Absorption of glass screen.

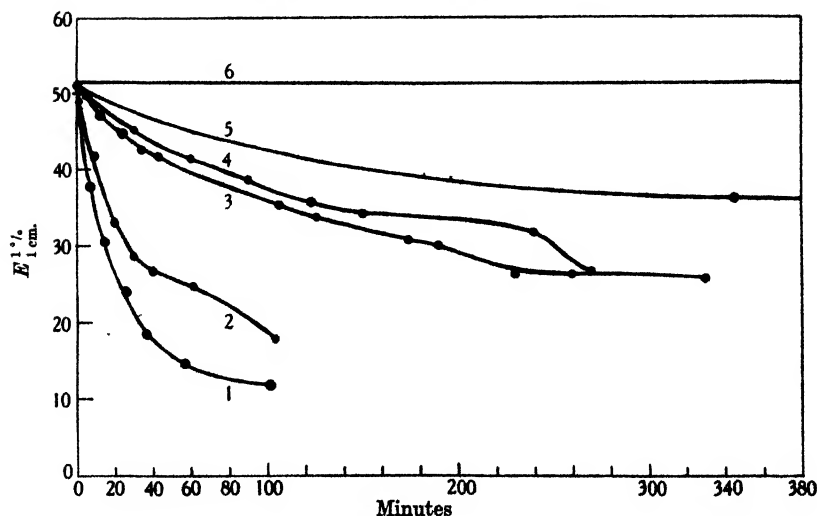


Fig. 2. Irradiation of fish liver oil No. 11/323. Curves 1-4 in cell with screen at following distances from mercury vapour lamp: (1) 20 cm.; (2) 26 cm.; (3) 35.5 cm.; (4) 45 cm. Curve 5, in diffused daylight, in specimen tube. Curve 6, in dark, in specimen tube.

0.5 to 25 sec. was normally used for each determination; the spectra were matched visually, and in every instance the accuracy of the match-points was checked by a second observer. The solution was transferred to the cell of the

spectrophotometer, and was irradiated either by exposure to the beam of light from the spark of the instrument, or by exposure to a mercury vapour lamp, after which the cell and contents were immediately transferred to the spectrophotometer for the absorption measurement. In order to minimize the effect due to light of very short wave-length, a glass microscope slide 1 mm. in thickness and having the transmission curve shown in Fig. 1 was interposed between the spark and the photometer in the first method and between the mercury vapour lamp and the cell in the second method. Fig. 2 shows a set of results obtained by irradiating an alcoholic solution of a fish liver oil with a mercury vapour lamp placed at different distances from the solution. It also shows the effect of

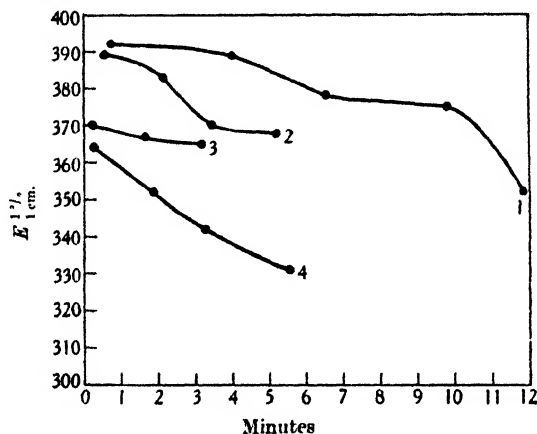


Fig. 3. Curve 1, irradiation of vitamin A concentrate No. 1/584 by tungsten-steel spark. Curves 2, 3 and 4, irradiation of same solution after recovery in the dark overnight.

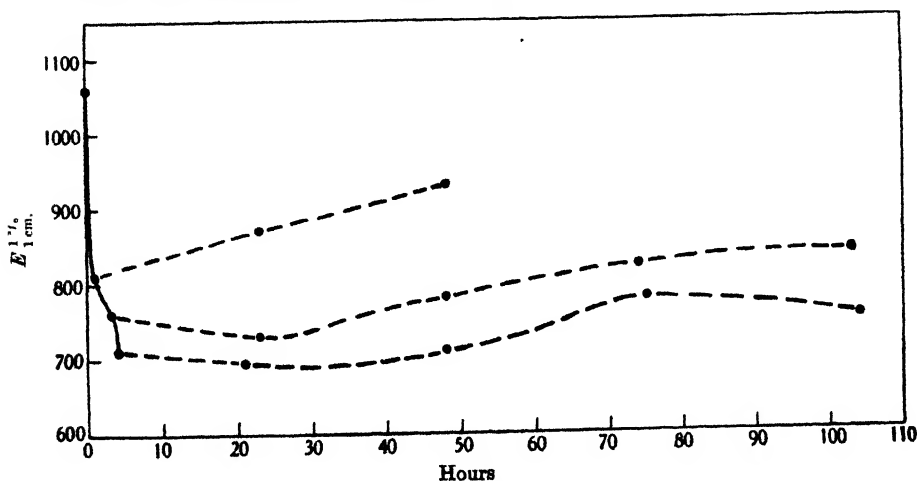


Fig. 4. Irradiation (—) and subsequent recovery (---) of concentrate No. S.A.I.

diffused daylight (from a north window) on the same solution. The same solution stored in the dark for over a month showed no appreciable change in the value of  $E_{1/2, 328 \text{ m}\mu}^{1/2}$ . Fig. 3 shows a set of similar results obtained by

irradiating an alcoholic solution of a concentrate with the spectrophotometer spark; it shows also the recovery to almost the original value that occurred on standing overnight in the dark, and the further decrease in  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  that took place on re-irradiating. The amount of the subsequent recovery was very small.

Fig. 4 illustrates the effect of irradiating an alcoholic solution of a concentrate with a mercury vapour lamp and shows that the degree of recovery diminishes with the period of irradiation. Fig. 5 A shows similar results, a point of particular interest being that the concentrate showed a preliminary increase in the value of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  when its solution was allowed to stand in the dark.

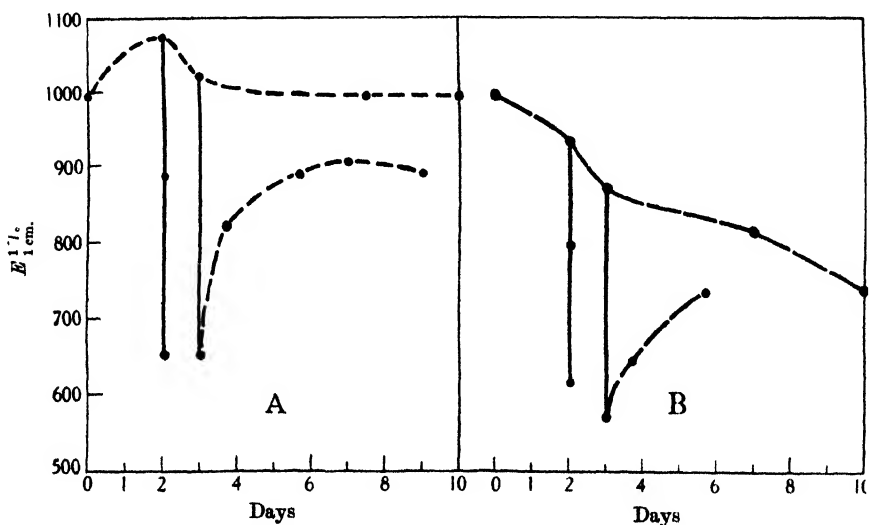


Fig. 5. Irradiation and recovery (A) in dark (B) in daylight of concentrate No. S.A.I.  
Irradiation —; Daylight — —; Dark . . . .

It is believed that previous exposure to daylight had reduced the value of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  and that the reversible change had taken place when the concentrate had subsequently been dissolved in alcohol and allowed to stand. It is suggested that this is also the explanation of the slight increase in the values of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  on storage of concentrates referred to above. Fig. 5B illustrates the slow decrease in absorption of the same concentrate on exposure to daylight and the rapid further decrease on exposure to the mercury vapour lamp, followed by slow recovery in daylight.

Fig. 6 shows the effect of irradiation, under identical conditions, on solutions of a vitamin A concentrate of the same concentration in different solvents. For ease of comparison the ordinates represent percentages of the initial values of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$ , because these values varied slightly in accordance with the solvent effect noted previously [Smith *et al.* 1938]. The reduction in the value of  $E_{1\text{ cm}}^{1\%}$  328  $m\mu$  on irradiation is also not independent of the nature of the solvent.

The effect of daylight may be of analytical importance, because errors may be introduced by exposure of the solutions to daylight during saponification of oils and extraction of the soap solutions. Table I shows the reduction in ultra-violet absorption when vitamin A solutions were allowed to stand in the light for a few hours. With some spectrophotometers long exposure of the vitamin

solution to the light from the spark or arc occurs during measurement of the absorption spectrum, and this may also cause errors, but the effect is negligible when only short exposures are given.

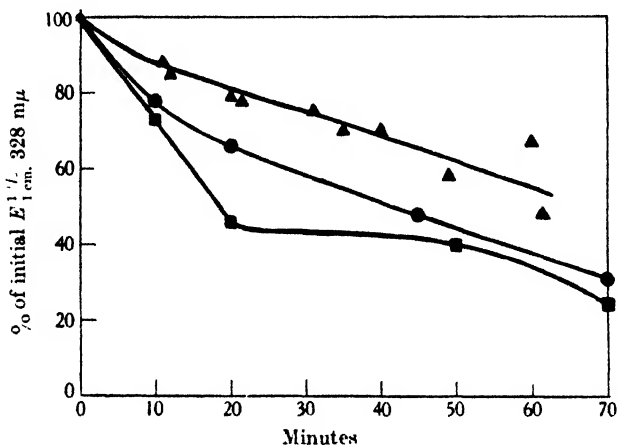


Fig. 6. Irradiation by mercury vapour lamp in various solvents.  
■ Cyclohexane. • Ethyl alcohol. ▲ Ether.

Table I. *Keeping properties of vitamin A in daylight*

Kept in corked flask on bench by north-facing window

No.	Type of sample	Solvent	Month	$E_{1\text{ cm}}^{1\text{ l.}} 328\text{ m}\mu$		Hr. exposure	% of initial $E$
				Initial	Final		
1/243	Concentrate	Cyclohexane	January	467	467	6	100
12/136	Halibut liver oil	Hexane	December	41.2	38.4	6½	93
12/168	Concentrate	Chloroform	January	405	387	7	96
S.A.I.	Concentrate	Cyclohexane	February	1030	821	47*	80
S.A.I.	Concentrate	Ether	February	1090	950	47*	87
S.A.I.	Concentrate	Chloroform	February	975	758	2	78
S.A.I.	Concentrate	Chloroform	February	900	915	2	95
					588	5	61
					85	22†	9
4/110	Concentrate	Cyclohexane	April	67.1	64.8	2	96.5
4/104	Concentrate (mammalian)	Cyclohexane	April	195	181	5½	93

\* Approximately 18 hr. of daylight.

† Approximately 8 hr. of daylight.

## DISCUSSION

It is impossible at present fully to explain these experiments. The most plausible hypothesis appears to be that already advanced [Smith *et al.* 1938]. It seems likely that the vitamin A in an oil or concentrate exists as a mixture of geometrical isomerides, but that on irradiation energy is absorbed, causing a change in the proportion of isomerides. It is very probable, as pointed out by Morton [1938], that *cis-trans* isomerides of vitamin A would display different intensities of absorption. On removal from the radiation slow reversion to the equilibrium mixture occurs. It is evident that there is a simultaneous irreversible photochemical change of the vitamin A into some other substance, possibly bicyclic, with little absorption at 328  $m\mu$ .



## SUMMARY

Exposure to ultraviolet radiations of wave-lengths exceeding  $300\text{ m}\mu$  approx. causes a progressive decrease in the value of  $E_{1\text{ cm}}^{1\%}$   $328\text{ m}\mu$  for vitamin A-containing concentrates or oils. This decrease is the more rapid the more intense the radiation, but even diffused daylight has some effect. When the irradiated solutions are allowed to stand in the dark (or in light of lower intensity) the value of  $E_{1\text{ cm}}^{1\%}$   $328\text{ m}\mu$  increases towards the original value. The further the value of  $E_{1\text{ cm}}^{1\%}$  has been reduced by irradiation, the less complete is the recovery in the dark. It appears that vitamin A suffers reversible photochemical isomerization. Simultaneously slow photochemical destruction of the vitamin occurs.

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# XXVI. PROLONGED ADMINISTRATION OF SEX HORMONES TO CASTRATED RATS

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OUR previous experiments with castrated male rats have been of only 23 days' duration [see references and technique, Korenchevsky, 1937; Hall & Korenchevsky, 1938, 2]. For obvious reasons the investigation of much longer periods of injections is important from both the experimental and the clinical viewpoints. In these experiments we have used the hormones which are known to occur naturally in males—androsterone, *transdehydroandrosterone* and testosterone. Oestrone also belongs to this group of "natural" hormones, but in this investigation oestradiol dipropionate, a more active compound with a prolonged activity [Miescher *et al.* 1938] has been used. For the same reason the activity of testosterone propionate has also been investigated.

## Technique

The hormones were administered for an average period of 105 days, the male hormones being injected 5 times a week, and oestradiol dipropionate 3 times a week, in the weekly doses indicated in Tables I and II. The total number of rats used for the experiments was 91, divided into 16 groups as shown in the Tables. The pure hormones were supplied by Messrs Ciba Ltd. and were dissolved in sesame oil, the single dose being injected in 0.2 ml. of the oil. The rats were castrated at the age of 22–25 days, i.e. before sexual maturity. The other details of our technique were the same as described in our previous papers.

Table I. *The effects of androsterone and transdehydroandrosterone injected alone or simultaneously with oestradiol dipropionate on actual weights of organs of castrated rats*

	I	II	III	IV	V	VI	VII	VIII
	Control rats (oil)		Oestradiol diprop.		Androsterone 7.5 mg.		Dehydroandrosterone 7.5 mg.	
	Normal	Castrated	0.018 mg.	0.090 mg.	Alone	+ 0.090 mg. oestrad. diprop.	Alone	+ 0.090 mg. oestrad. diprop.
Seminal vesicles (mg.)	1309	12	131	129	403	153	39	74
Prostate (mg.)	1341	62	142	144	981	791	132	219
Penis (mg.)	364	98	132	140	289	210	158	163
Preputial glands (mg.)	128	38	65	47	149	117	133	84
Adrenals (mg.)	53	74	68	61	50	78	56	62
Hypophysis (mg.)	10.0	12.1	49.1	75.4	9.9	56.8	12.8	45.7
Thymus (mg.)	241	364	227	183	299	230	381	185
Liver (g.)	13.13	10.43	9.60	9.29	14.60	12.53	12.35	8.59
Kidneys (g.)	2.42	1.89	2.00	1.88	2.53	2.67	2.08	1.62
Spleen (mg.)	803	646	593	528	1022	863	973	607
Heart (mg.)	1135	950	868	833	1236	979	1066	785
Retroperitoneal fat (g.)	16	20	10	10	24	17	17	7
Final body weight (g.)	437	377	259	254	437	334	381	225
Gain in body weight (g.)	275	243	128	95	249	132	254	111
No. of rats in group	14	13	4	6	4	4	4	4

Table II. *The effects of testosterone and testosterone propionate injected alone or simultaneously with oestradiol dipropionate on actual weights of organs of castrated rats*

	IX Testosterone 0.75 mg.		X Testosterone 0.75 mg.		XI Testosterone 7.5 mg.		XII Testosterone 7.5 mg.		XIII Testost. prop. 0.75 mg.		XIV Testost. prop. 0.75 mg.		XV Testost. prop. 7.5 mg.		XVI Testost. prop. 7.5 mg.	
	+ 0.018 mg. oestradiol diprop.		+ 0.090 mg. oestradiol diprop.		+ 0.018 mg. oestradiol diprop.		+ 0.090 mg. oestradiol diprop.		+ 0.018 mg. oestradiol diprop.		+ 0.090 mg. oestradiol diprop.		+ 0.018 mg. oestradiol diprop.		+ 0.090 mg. oestradiol diprop.	
	Alone		Alone		Alone		Alone		Alone		Alone		Alone		Alone	
Seminal vesicles (mg.)	95	254	812	1216	1231	1097	2526	2363	217	405	720	960	1322	1161	2078	2087
Prostate (mg.)	216	227	368	338	346	301	414	379	59	82	112	100	142	136	198	166
Penis (mg.)	56	75	58	56	57	84	62	77	13.7	30.1	11.9	64.5	11.7	36.1	9.6	21.0
Preputial glands (mg.)	275	165	234	107	220	203	95	54	Adrenals (mg.)	56	75	58	56	57	84	62
Hypophysis (mg.)	10.62	10.94	11.07	8.69	12.95	10.56	11.15	8.80	Thymus (mg.)	275	165	234	107	220	203	95
Liver (g.)	1.87	2.31	1.98	1.78	2.35	2.41	2.59	3.19	Liver (g.)	10.62	10.94	11.07	8.69	12.95	10.56	11.15
Kidneys (g.)	870	805	805	666	952	855	803	755	Kidneys (g.)	1.87	2.31	1.98	1.78	2.35	2.41	2.59
Spleen (mg.)	954	939	1016	740	1152	1021	1127	940	Spleen (mg.)	870	805	805	666	952	855	803
Heart (mg.)	13	11	12	9	17	11	9	5	Heart (mg.)	954	939	1016	740	1152	1021	1127
Retroperitoneal fat (g.)	339	281	348	239	419	337	335	255	Retroperitoneal fat (g.)	13	11	12	9	17	11	9
Final body weight (g.)	229	175	208	97	275	185	177	82	Final body weight (g.)	339	281	348	239	419	337	335
Gain in body weight (g.)	4	4	4	4	6	5	7	4	Gain in body weight (g.)	229	175	208	97	275	185	177
No. of rats in group									No. of rats in group	4	4	4	4	6	5	7

For economy of space the weights per unit (200 g.) of body weight are not given, but they will be mentioned in the text whenever they alter the conclusion based on actual weights only.

Detailed histological results will be published elsewhere. In this paper are given only short preliminary statements without which the interpretation of the results would be difficult.

Oestradiol dipropionate will be abbreviated in the text to "oestradiol".

#### *Effect on secondary sex organs*

The effects of the prolonged injections on the seminal vesicles, prostate, coagulating glands, penis and preputial glands, atrophied after castration, were in some cases similar to, in others different from, those obtained in our previous experiments of shorter duration.

*Male hormones.* On the basis of the results of the present experiments the male hormones could be grouped in the following descending order of activity—testosterone propionate, testosterone, androsterone and dehydroandrosterone: only one hormone, testosterone propionate, was able to restore to normal (col. XIII), or, in large doses to produce supernormal (col. XV), development of seminal vesicles and prostate; the activity of androsterone was much more pronounced on the prostate than on the seminal vesicles (col. V). All these results were similar to those obtained in our 23-day experiments.

The prolongation of the injections strengthened the effect of androsterone, testosterone and testosterone propionate, but not that of dehydroandrosterone, which remained very weak, as comparison of the figures of Tables I and II with the data given in our previous papers [Korenchevsky *et al.* 1935–37] shows.

A close correlation was present between the degree of restoration towards normal of the weight and size of the organs and their histological structure. No pathological changes were observed, except for some abnormal vacuolation of the epithelial cells in the central and lateral lobes of the prostate in the rats injected with large doses of testosterone propionate.

*Oestradiol dipropionate* (cols. III and IV) increased the weight and size of the seminal vesicles to a degree remarkable for a female hormone, having a stronger effect on these organs than dehydroandrosterone. Even a definite hypertrophy of the penis was obtained, which has not previously been found with pure oestradiol or oestrone. The effect on the preputial glands, the weights of which vary considerably both in control and injected animals, was less definite. Increasing the dose from 0.018 mg. (col. III) to 0.090 mg. (col. IV) did not increase the effect on the male sex organs.

However, this remarkable effect of oestradiol on the weight and size of the seminal vesicles and prostate was not accompanied by restoration of the secretory epithelium, but was chiefly due to pathological development of fibrous tissue in these organs, while after shorter periods of injections [Korenchevsky & Dennison, 1935] the muscular layer was also greatly hypertrophied. The penis of the "oestradiol" rats showed better development of the corpora cavernosa and also partial separation of the epithelial layers of the preputium and of the glans penis, which are fused in castrated control rats. The epithelial layers showed slight development, but still resembled those in castrated control rats and not those in normal animals.

*Co-operative or antagonistic activities of male and female hormones and the neutralizing effect of the male hormones on the pathological changes caused by the oestrogens*

As judged by the weights of the organs, testosterone and oestradiol showed strong co-operative activity (cols. X and XII). This is in agreement with the results of our previous experiments of 23 days' duration [Korenchevsky *et al.* 1936]. This co-operative activity was confirmed histologically, especially with the large dose (col. XII); with the small dose of testosterone (col. X) the pathological effects of the oestradiol were not completely neutralized and were evident in the excessive development of fibrous tissue and stratification of the epithelium, which was, however, of the more normal columnar type. Testosterone propionate showed no co-operative activity with oestradiol (cols. XIV and XVI) in respect either of weight or histological structure; with both doses of testosterone propionate, the pathological effect of the oestradiol was seen only occasionally and in a slight degree.

The apparent absence of a co-operative activity of testosterone propionate with oestradiol is not due exclusively to the fact that the male hormone alone, in the doses used, produces normal or even supernormal development of the sex organs, so that its activity with the oestrogen is veiled. This conclusion is supported by the fact that in our previous experiments with small doses of testosterone propionate [Korenchevsky & Dennison, 1937] a similar result was obtained (only very slight effect on seminal vesicles). Slightly smaller sex organs in the rats injected with both hormones (cols. XIV and XVI) as compared with those injected with the male hormone alone (cols. XIII and XV) may be explained by the smaller size of the former rats; no such difference was seen in the weights of the organs calculated per unit of body weight.

An antagonistic effect was unexpectedly obtained when androsterone was injected simultaneously with oestradiol dipropionate (col. VI). This effect was very pronounced on the seminal vesicles (both in actual weights and those per unit of body weight), but less so on other sex organs (noticeable only in weights per unit of body weight). This antagonistic effect is the more remarkable since with smaller doses injected for shorter periods [Korenchevsky *et al.* 1935] a

definite co-operative effect of androsterone with oestrone was obtained both by weight and (unpublished data) histologically.

When *trans*dehydroandrosterone was injected simultaneously with oestradiol the weight of the seminal vesicles (74 mg. col. VIII) was less than that in rats injected with oestradiol alone (129 mg. col. IV). In this case, therefore, the male hormone exercised a definite antagonistic effect on the activity of the oestradiol. This interaction was not present in the case of the prostate and penis. Histological investigation confirmed this antagonistic activity of oestradiol with androsterone or dehydroandrosterone, at the same time revealing some neutralizing effect of these male hormones on the pathological changes produced by the oestrogen. Thus, although fibrous tissue was excessively developed and the epithelium was stratified (antagonistic "oestrogens" effect), the fibrosis was much less pronounced than with oestradiol alone, and the epithelium was of the more normal columnar type (neutralizing "male" effect).

*Comparison with the effects of the same hormones on normal, non-castrated rats*

In our experiments of long duration with non-castrated rats [Korenchevsky & Hall, 1939] it was unexpectedly found that the development of the secondary sex organs of these normal animals was depressed by androsterone, not significantly changed by testosterone and considerably stimulated by testosterone propionate. These remarkable differences in the activities of the male hormones were explained by the balance of two simultaneously exercised, opposite activities: (a) the depressing effect of the injected male hormones on the anterior lobe of the hypophysis, resulting in a more or less decreased secretion of gonadotropic hormones, which in turn decreases the secretion of the natural male hormones from the testes. This should produce atrophy of the secondary sex organs, but (b) this effect is counterbalanced by the stimulating effect produced directly, but in different degrees, by the injected male hormones on the secondary organs. In the case of the weaker sex hormones (androsterone and *trans*dehydroandrosterone) the atrophying effect (a) prevailed, but with testosterone propionate the hypertrophying effect (b) was the stronger. With testosterone, a hormone of medium strength, the two activities neutralized one another and therefore no change was obtained in the condition of the sex organs.

This mechanism cannot operate in the case of gonadectomized animals, and the results of our present experiments on castrated animals corroborate this: all the male hormones, without exception, produced stimulation of the secondary sex organs, the degree of stimulation produced corresponding to the strength of their sexual activity. The varying results obtained when the different male hormones were injected simultaneously with oestradiol probably depended upon the strength of the particular male hormone as compared with oestradiol. Therefore the different male hormones, when injected simultaneously with oestradiol into castrated animals, were divided according to their different degrees of activity into 3 groups, each consisting of the same hormones as in the case of normal (non-castrated) rats injected with the male hormones alone.

*Effects on non-sexual organs, fat deposition and body weight*

*Effect of castration.* We have described in our previous papers [Korenchevsky and co-workers, see references 1934-37] how castration produces more or less pronounced changes in the various non-sexual organs of the rat. The results obtained have been confirmed by our present experiments and may be summarized as follows (compare cols. I and II, Table I): varying degrees of hypertrophy of adrenals and hypophysis; delayed involution (i.e. increased size) of

the thymus; decrease in the weight of the liver, kidneys, heart and spleen (a definite change of the spleen recorded only in the present long-duration experiments); decreased growth and gain in body weight, but increased fat deposition; no definite changes in the thyroids. When calculated per unit of body weight, these changes are even more striking in the adrenals, hypophysis and thymus, but less pronounced in the other organs. Therefore a certain amount of the decrease in weight of liver, kidneys, spleen and heart corresponds to and can be explained by the smaller size and weight of the castrated animal. Part of this "oligosplanchnia" should, however, be explained by the specific effect of gonadectomy, i.e. by the absence of secretion of the testicular hormones. Specific histological changes after castration are well known and have been found in the hypophysis by several workers and in the adrenals by Deanesly [1928], Andersen & Kennedy [1933], Deanesly & Parkes [1937], Cramer & Horning [1937], Hall & Korenchevsky [1937; 1938, 1]. In the liver only a slight decrease in the size of the lobules has been observed [Hall & Korenchevsky, 1938, 2]. Histological examination of the other organs investigated in the present experiments has not yet been completed.

#### *Effects of the hormones*

*Gain in body weight.* The figures given in the Tables for the gain in body-weight during the experiment, taken in conjunction with the figures for the final body weight, strongly indicate an effect of the sex hormones upon metabolism. While oestradiol (cols. III and IV) produces a pronounced decrease in body weight resulting in stunted growth, with the male hormones this depressing effect is absent (androsterone, *trans*dehydroandrosterone, small doses of testosterone and testosterone propionate, cols. V, VII, IX, XIII) or less pronounced (large doses of testosterone and testosterone propionate cols. XI and XV). Moreover, this depressing effect of oestradiol can be partly neutralized by the simultaneous injection of male hormones (cols. VI, VIII, X, XIV) except in the cases of large doses of testosterone and testosterone propionate (cols. XII and XVI). These exceptions are interesting and important in that they demonstrate qualitatively different effects of different doses, which property is well known in pharmacology for several compounds and in endocrinology is especially important in the case of simultaneous injections of oestrogens and progesterone.

That the effect on the body weight is dependent upon the size of the dose is in most cases confirmed by comparison of the data of our present experiments with the results previously obtained. When injected for shorter periods (i.e. total dose being smaller), androsterone [Korenchevsky *et al.* 1935], testosterone [Korenchevsky *et al.* 1936] and small doses of testosterone propionate [Korenchevsky *et al.* 1937] caused increased gain in body weight, while large doses of testosterone propionate gave the same decrease, and dehydroandrosterone the same absence of effect both in long and short duration experiments.

A definite neutralizing property on the depressing effect of oestradiol is noteworthy in the weak sex hormones, androsterone and even to some extent dehydroandrosterone.

*Fat deposition.* With the exception of androsterone all the hormones investigated (including oestradiol) decreased, in varying degree, the amount of fat deposited. The depressing effect of oestradiol on the deposition of fat was considerably neutralized by simultaneous injections of androsterone, but not affected by small doses of testosterone and testosterone propionate, while dehydroandrosterone and large doses of testosterone and testosterone propionate when injected simultaneously with oestradiol caused an even greater loss of fat.

With shorter periods of injections (see references mentioned above), androsterone, dehydroandrosterone and testosterone produced no change in the fat deposition, while testosterone propionate decreased it as in the present prolonged experiments.

**Adrenals.** The hypertrophied "castration" adrenals (col. II), as judged by weight, both actual and per unit of body weight, returned to or towards normal (cols. V, VII, IX, XI, XIII and XV) after prolonged injections of male hormones, the changes both in weight and histologically being similar to those previously obtained in experiments of 3 weeks' duration [Hall & Korenchevsky, 1937; 1938, 1]. Large doses of testosterone propionate caused abnormal depletion of lipoid from some parts of the cortex. It is remarkable, however, that the longer period of injections did not, as one would theoretically expect, cause greater, but rather less pronounced, changes, as though some kind of neutralizing mechanism had developed during the prolonged administration of the male hormones.

When oestradiol was injected alone, the actual weight of the "castration" adrenal was slightly decreased but, when the weights are calculated per unit of body weight, a definite increase is observed with both doses (an average of 53 and 48 mg. in the injected rats as compared with 38 mg. in the control castrated animals). Evidently the weight of the adrenals was only slightly decreased in correlation with the stunted growth of the "oestradiol" rats, but the effect of the oestradiol on the adrenals is essentially hypertrophying. This conclusion is corroborated in a different way in most of the groups in which male and female hormones have been injected simultaneously (cols. VI, VIII, X, XIV, XVI): in these cases an abnormal hypertrophy of the adrenals occurred even in actual weights. A similar interrelation has been recorded in experiments of 3 weeks' duration [Korenchevsky and co-workers, 1935-37]. Previously also in experiments of shorter duration, Korenchevsky & Dennison [1934] found that oestrone produced hypertrophy in normal, but not in castrated, rats.

Histologically, oestradiol produced hyperaemia, a narrower *zona glomerulosa* with some atrophy of its cells, patches of peculiar amyloid-like degeneration in the *zona reticularis*, and the appearance of "nests" of specific cells at the boundary between the cortex and the medulla.

These "oestradiol" changes in the adrenals were not completely neutralized by the simultaneous injection of male hormones, thus corroborating the results obtained by weights.

**Hypophysis.** It is now well established that oestrogens produce enlargement of the anterior lobe of the hypophysis, resulting, with sufficiently large doses, in a tumour-like hyperplasia of the gland. In our present experiments we also have obtained similar changes in weight (Table I, cols. III and IV) and size of the hypophysis.

Male hormones alone, in both our previous and present experiments and in those of other workers, had no considerable or constant effect on the size and weight of the hypophysis. Allanson [1937] has given a good review of the literature on the subject and added her own histological results.

In our previous experiments of 3 weeks' duration, the weight of the "castration" hypophysis was decreased to normal only after injections of androsterone. In the present long-duration experiments this recovery was effected by androsterone (col. V) and also by large doses of testosterone propionate (col. XV).

It is necessary to emphasize the most important result that all the male hormones used were able to prevent to some extent development of the hypophysial hypertrophy caused by oestradiol, the greatest effect being produced

by the large dose of testosterone propionate (col. XVI, average weight 21 mg. as compared with 75.4 mg. in the rats injected with oestradiol alone). In one of the rats of this group (testosterone propionate + oestradiol) the hypophysis weighed 14 mg., which weight is within the variations present in the control uninjected groups.

Wolfe & Hamilton [1937], in experiments of 10 days' duration, also noticed some suppression of oestrogenic changes in the hypophysis by male hormones. Thus a definite antagonistic interrelation between male and female hormones is apparent in their effects on the hypophysis.

*Thymus.* All the hormones, except dehydroandrosterone, hastened the involution of the thymus, and the simultaneous injection of oestradiol and male hormones had a co-operative effect, these results being similar to those obtained in our shorter-duration experiments (see references already mentioned).

*Liver, kidneys, spleen and heart.* From a study of the data given in Tables I and II, it is possible to come to a general conclusion that male hormones cause an increase, and oestradiol (except in kidneys) a decrease, in the weights of these organs in most rats. Dehydroandrosterone and large doses of testosterone and testosterone propionate appear to increase this "oligosplanchnic" effect of oestradiol. Androsterone, on the contrary, is able to neutralize this effect, even when large doses of oestradiol are given, while in the cases of testosterone and testosterone propionate this neutralization occurs only with small doses of oestradiol. However, for true megalo- or oligo-splanchnia, not only the actual weights, but also the weights per unit of body weight should show the same change, i.e. the changes in weight of the organs should be at least partly independent of the changes in weight and size of the animals. If the changes we have obtained are considered from this point of view, and the weights per unit of body weight (not included in the Tables) are also taken into consideration, the following conclusions are reached:

- (1) The male hormones can be considered as true stimulators of certain organs in castrated rats, but not in normal animals [cf. Korenchevsky & Hall, 1939]. In other words, they restore the size and weight of the liver, kidneys, spleen and heart to or towards normal, but are unable to cause supernormal enlargement.

- (2) It is remarkable that this stimulating activity should be most strongly manifested, not by the most sexually active hormone, testosterone propionate, but by the relatively weak sex hormone, androsterone.

- (3) The female hormone, oestradiol dipropionate, can be regarded as a stimulator only when the weights are calculated per unit of body weight (e.g. these weights of liver, kidneys and heart in rats injected with oestradiol were respectively 7.4, 1.5 and 0.66 g. as compared with 5.5, 1.0 and 0.49 g. in castrated control rats).

- (4) Since the body fat in the "oestradiol" rats is much decreased, a comparison with the much fatter control animals is difficult. From the point of view of metabolism, one unit of passive fat tissue requires much less activity of the organs regulating metabolism (liver, kidneys, endocrines etc.) than do the more active tissues (e.g. muscular, glandular, nervous etc.). Therefore in fat animals one would expect that the weights of these "metabolism" organs, when calculated per unit of body weight, should be smaller than in animals in which the amount of body fat is small, provided that the metabolic rate is about the same.

According to Reed *et al.* [1930] the amount of genital and retroperitoneal fat in rats represents about 32% of the whole adipose tissue in the body. According to our observations, the amounts of these two types of fat (as



dissected by us) are about equal (i.e. 16 %). If in all groups of our rats the total body fat is calculated on this basis and subtracted from the final body weight, the remaining weight will represent, at least chiefly, the approximate weight of the active tissues in the body. Even with this correction the weights per unit of body weight of the liver, kidneys and heart are heavier in the oestradiol rats than in the castrated control animals (9.8, 2.0 and 0.88 g. respectively, as compared with 8.5, 1.5 and 0.75 g.). It must, however, be admitted that this correction is neither perfect nor complete, since the amount of fat present in the cells of the organs is not taken into consideration.

Macroscopically it was noticed that the livers of castrated control rats were light brown in colour, sharply differing from the dark, reddish-brown livers of the "oestradiol" rats. Preliminary histological investigation has shown that this difference in colour is due partly to hyperaemia in the livers of the oestradiol rats and partly to the much reduced amount of fat and lipoids in the liver cells of these animals as compared with those of castrated controls. These changes were present also in the livers of rats injected simultaneously with oestradiol and male hormones. No pathological changes were found in the livers after injections of male hormones alone, such livers appearing normal.

The changes in weight of the organs and the results of preliminary histological investigation of the liver and kidneys suggest that the male sex hormones most probably have a favourable stimulating effect upon the liver, kidneys, heart and spleen of castrated rats. At present the true nature of the activity of oestradiol on these organs is difficult to define until histological investigation is completed. The changes per unit of body weight, however, especially in the case of kidneys, may possibly indicate that oestrogens also have some stimulating effect upon these organs, veiled, however, by the stunted growth of the organism and fat impoverishment of the body.

#### *Comparison of the effects on non-sexual organs with those on sexual organs*

Two important points must be emphasized in the nature of the reaction of the different organs to castration and injections of the sex hormones:

(1) Castration and the injection of sex hormones into gonadectomized rats always cause typical changes in the sexual organs of every animal, while, in most cases, the weights of the non-sexual organs, fat deposition and gain in body weight are changed only in the majority of animals, the average figures, however, showing typical changes. If, however, the effects produced by the hormones are studied within the same litter, the rats of which are divided into the different groups, the number of animals showing the same typical changes is much increased, in several cases the exceptions being absent. The importance of comparison with control animals of the same litter has been emphasized in our previous papers [Korenchevsky, 1932; Korenchevsky *et al.* 1932; Korenchevsky & Dennison, 1934]. However, in the present experiments comprising 16 groups this was impossible, as our litters consisted of 4-8 rats only, which were divided into as many groups as possible.

(2) While prolongation of the injections of all hormones, except dehydroandrosterone, caused an increase in the effect upon the sexual organs, the effect upon the non-sexual organs was not increased, but even in some cases (e.g. testosterone) decreased. Probably this difference in reaction is at least partly to be explained by the changes produced in the gain in body weight and, perhaps, fat deposition, by the prolongation of the injections. As described above, the changes in the liver, kidneys, heart and spleen are much more dependent on the body weight than are those in the sexual organs.

## SUMMARY

1. The effects upon the sexual and some other organs of prolonged administration (about 3½ months) of sex hormones to castrated rats are described and compared with the results obtained in our previous experiments of shorter duration (about 23 days). The hormones injected were oestradiol dipropionate, androsterone, *trans*dehydroandrosterone, testosterone and testosterone propionate injected alone, or oestradiol dipropionate combined with the male hormones.

*Effects on sexual organs*

2. Prolongation of the injections was followed by a greater effect on the sexual organs in the case of androsterone, testosterone and testosterone propionate, but not of *trans*dehydroandrosterone.

3. Testosterone propionate was the only hormone which produced complete restoration of the atrophied sexual organs, or, with large doses, a great super-normal development of the seminal vesicles and prostate.

4. A comparatively small dose of oestradiol dipropionate (0.018 mg. per week) produced (for a female hormone) a great enlargement of the seminal vesicles, coagulating gland, prostate and even penis, but increasing the dose did not increase the effect.

5. As with other oestrogens, this apparent return towards normal weight and size of the sexual organs was of pathological nature and caused chiefly by increased development of fibrous tissue. This fibrosis should, however, be considered as one of the specific sex effects, as it was present only in the sexual organs.

6. With the doses used oestradiol dipropionate showed pronounced co-operation in restorative activity with testosterone, but not with testosterone propionate, while an antagonistic interaction with androsterone and *trans*dehydroandrosterone (in contrast to the co-operative effect of androsterone with the weaker compound, oestrone) was found.

7. The neutralizing effect of the male hormones on the pathological changes in the sexual organs, produced by as strong an oestrogen as oestradiol dipropionate, was very pronounced in the case of testosterone and testosterone propionate, but weak with androsterone and *trans*dehydroandrosterone.

*Body weight and fat deposition*

8. Only oestradiol and, to a lesser degree, large doses of testosterone and testosterone propionate caused a decrease in the gain in body weight. Androsterone, *trans*dehydroandrosterone and small doses of testosterone and testosterone propionate, although having no effect when injected alone, partly neutralized the depressing effect upon growth of the simultaneously injected oestrogens.

9. All the hormones in the doses used caused in varying degree a decrease of the amount of body fat, except androsterone, which was even able in this respect to neutralize the effect of oestradiol.

*Non-sexual organs*

10. All the hormones accelerated the physiological involution of the thymus and there was a co-operative relation in this respect between the male hormones and oestradiol dipropionate.

11. The male hormones prevented to a considerable degree (with testosterone propionate in some cases almost completely) the tumour-like hyperplasia of the hypophysis produced by oestrogens.

12. The male hormones in most cases caused the "castration" adrenal to return to or towards normal, while oestradiol dipropionate produced specific pathological changes in this gland which were only partly neutralized by simultaneous injections of the male hormones.

13. Slight hypertrophy of liver, kidneys, heart and spleen was produced by the male hormones in most rats but oestradiol dipropionate caused either a decrease in their actual weights or (kidneys) no change, "dark" liver being a constant feature of all rats injected with this hormone.

14. This "oligosplanchnic" effect of oestrogens was to some extent neutralized by the male hormones, the comparatively weak sex hormone, androsterone, having the greatest effect in this respect.

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## XXVII. GLUTAMINE AND THE GROWTH OF *STREPTOCOCCUS HAEMOLYTICUS*

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IN the course of our analysis of the nutrients of *Streptococcus haemolyticus* it was found that growth did not take place in a mixture containing peptone, cystine, glucose, phosphate and a number of other substances until an extract of meat also was added. Good growth then took place in some 16 hr. whereas in the culture without meat extract no growth was visible during the period of observation, usually 2 days but often 3 or 4 and on one occasion 9 days.

The test coccus was the well-known strain "Richards" maintained at high virulence by repeated passage through mice and kept in the intervals in rabbit blood. For the purposes of this work it was transferred from the blood to nutrient agar as required for the tests. After a variable number of subcultures on agar it was found that the coccus was of no further use as a test object in that it became able to grow on the peptone mixture without our extract of meat. This phenomenon of alteration in nutrient requirements we ascribed to the development of a power to synthesize our meat extract factor which was lacking while the organism was maintained in blood. Since the power to synthesize our factor was thus potentially present, positive results obtained by the addition of some fractions of meat, which were obtained only after 3 or 4 days' incubation, were suspect even though the control without the addition might be negative. In such cases it was possible that the fraction of meat did not contain the active substance we were seeking but did contain (a) a related compound from which our active substance was synthesized, or (b) some not necessarily specific substance which accelerated the enzymic processes utilized in the synthesis. In order therefore to ensure a test object which was incapable of growth in the absence of our meat extract we used a culture which was recovered from the blood every 7 days; and to satisfy ourselves that we were titrating our active substance itself and not something else which facilitated its synthesis we judged the potency of meat fractions by readings of growth up to 24 hr. and not 2 or 3 days.

### *Bacteriological technique*

The general technique used on the bacteriological side of this work has followed that customary in this laboratory in nutritional studies of bacteria. The inoculum used has been relatively small. A small mass of bacteria was raked from an agar surface in such a way as to remove none of the medium and transferred to water. This was thoroughly suspended with a Pasteur pipette and diluted until the suspension was just invisible to the naked eye. One drop of this was used per tube. A precise enumeration of the number of cocci in a

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streptococcal suspension is impracticable but the number of colonies which grew on plating out the inoculum was usually about 1 million. It may, however, be stated that the strain used by us was capable of growing in the mixture to be described when the inoculum was reduced approximately to one coccus.

The inadequate basal medium used for the demonstration of the activity of the meat extracts was made as follows:

Bacto peptone (Difco)	10.0 g.
NaCl	5.0 g.
$\text{KH}_2\text{PO}_4$	4.5 g.
<i>N</i> NaOH	26 ml.
Water	to 700 ml.

Adjust to pH 7.4; distribute in 7 ml. lots in test tubes (6 in.  $\times$   $\frac{3}{4}$  in.) and autoclave. At the time of testing add to each tube:

Sample to be tested (filtered)	1 ml.
<i>M</i> /18 phosphate buffer pH 7.4 (autoclaved)	1.05
<i>M</i> /20 cystine HCl in <i>N</i> /10 HCl (filtered)	0.2
<i>N</i> /5 NaOH (autoclaved)	0.25
Mixture <i>A</i>	0.5
Inoculum	1 drop

Mixture *A* consisted of:

Aneurin	0.005 g.	Thymine	0.01 g.
Nicotinamide	0.005 g.	Guanine	0.01 g.
$\beta$ -Alanine	0.005 g.	$\text{KH}_2\text{PO}_4$	0.3 g.
Pimelic acid	0.05 g.	Glucose	5.0 g.
Uracil	0.01 g.	Riboflavin <i>M</i> /5000,	20 ml.
Cytosine	0.01 g.		

Uracil, cytosine, thymine and guanine were dissolved in 50 ml. *N*/10  $\text{H}_2\text{SO}_4$  and neutralized with NaOH to pH 6.0. The other ingredients were then added and water to 90 ml. Readjusted to pH 6.0 and made up to 100 ml. Filtered Seitz EK.

The tubes were incubated in an upright position at 37° in air + 5%  $\text{CO}_2$ .

With regard to the constituents of mixture *A*, glucose is, of course, necessary as a source of energy but the part played by the others in growth has not yet been determined. In the absence of all, growth is usually but not always absent during the first day, but it may occur on the second day.

#### *Chemical fractionation of meat*

*Extraction.* The sodium sulphate method of Deutsch *et al.* [1938] was applied to fresh horse meat. The fat was cut from meat (20 lb.); the flesh minced (7.38 kg.; water content, 75%), mixed with anhydrous  $\text{Na}_2\text{SO}_4$  (2.77 kg.;  $\frac{1}{2}$  of the weight of water in the tissue) and the mixture twice remixed to a homogeneous stiff brown paste. This was heated with frequent stirring in a water bath at 45–50°; its temperature rose to 32° in the first 15 min. and remained so for a further 15 min. during the dissolving of the  $\text{Na}_2\text{SO}_4$ . The paste then became sloppier and its temperature rose. It was expressed at 35°; the extract cooled to room temperature;  $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$  filtered off; the mother liquors cooled to 0° and more  $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$  removed, leaving 1160 ml. of meat juice at about  $2\frac{1}{2}$  times its concentration in the tissues and containing about 4%  $\text{Na}_2\text{SO}_4$ . In several

batches the yields ranged from 140 to 180 ml./kg. of fresh meat or about 50–60 % of the amount theoretically obtainable from the water content of the tissue. The efficacy of the various methods of separation attempted was judged by determination of the total N and growth activity of the fractions. Results obtained with the fractions described are given in Table III (p. 228).

*First mercury precipitation.* Mercuric acetate precipitated only part of the activity in the  $\text{Na}_2\text{SO}_4$  extract; precipitation was complete under Neuberg's mercuric acetate-carbonate-alcohol conditions, but an equally complete precipitation was obtained with mercuric acetate in neutral 60 % alcoholic solution.

The extract (1000 ml.) was mechanically stirred and excess of finely powdered mercuric acetate (c. 100 g.) added, the solution neutralized with 10*N* NaOH (c. 60 ml.), alcohol (1500 ml.) added and the whole kept 2 hr. The precipitate was collected, freed from associated material by washing 3 times with water (500 ml.), stirred in water (500 ml.) in a closed jar and decomposed by  $\text{H}_2\text{S}$  under slight pressure. The  $\text{HgS}$  was filtered off and washed with water. The filtrate and washings from the sulphide contained almost the whole of the activity of the original extract and when evaporated in a vacuum gave a gum which retained its activity on keeping.

*Phosphotungstic acid precipitation.* Mercury-precipitated material equivalent to 1000 ml. of original extract was dissolved in 2*N*  $\text{H}_2\text{SO}_4$  (300 ml.) and 20 % phosphotungstic acid in 2*N*  $\text{H}_2\text{SO}_4$  added gradually with continuous stirring till in excess (c. 650 ml.). The precipitate was filtered after 4 hr. and washed 3 times with 2*N*  $\text{H}_2\text{SO}_4$  containing a little phosphotungstic acid.  $\text{H}_2\text{SO}_4$  and excess phosphotungstic acid were removed from the combined filtrate and washings, with baryta (c. 420 g.), excess Ba precipitated with  $\text{H}_2\text{SO}_4$  and the solution filtered and evaporated under reduced pressure to 30 ml. Inorganic salts separated on keeping and were filtered off.

*Properties of meat concentrates.* The active material was not removed from aqueous solution by a wide variety of adsorbents and immiscible solvents at a variety of pH. In the  $\text{Na}_2\text{SO}_4$  extract it was stable at 0° for several weeks. It was not very stable to heat: a little loss occurred at pH 5, 6 and 7 during heating for 1 hr. at 100° and  $\frac{1}{2}$  of the activity was lost in 3 hr.; losses were slightly greater in all cases at pH 8 and 9. It was stable to mild oxidizing and reducing agents (air, silver salts;  $\text{H}_2\text{S}$ ,  $\text{SO}_2$ ). The following indications of its amino-acid nature were obtained: it was more completely precipitated by  $\text{Hg}(\text{CH}_3\text{CO}_2)_2\text{-Na}_2\text{CO}_3$  in aqueous solution than by  $\text{Hg}(\text{CH}_3\text{CO}_2)_2$  itself: it yielded a moderately insoluble copper salt which was not, however, useful in separation: it was destroyed by nitrous acid at room temperature in dilute aqueous solution and by alloxan during 10 min. at 100°.

#### *Observations on glutamic acid and glutamine*

At this point we observed the note by Rane & Subbarow [1938] in which they described the growth of *Str. haemolyticus* in a gelatin-hydrolysate medium containing, in addition to other substances, glutathione which they considered to be a "significant" constituent of the nutrients. We tested glutathione in the concentration suggested in combination with various constituents of our mixtures not referred to here and in particular as a replacement of our meat extract factor, and in the latter circumstances found that it gave a trace of activity, which trace was increased by increase in the concentration of glutathione. The activity of our meat extracts could not however be due to glutathione for the following reasons:

- (1) Our active concentrates contained no demonstrable SS—SH group.

(2) Cuprous oxide, under the conditions described by Hopkins [1929] for glutathione, precipitated from our concentrates a small quantity of cuprous salts which contained only a trace of the original activity.

(3) Glutathione in concentrations equal in activity to our purer preparations, which were however still impure, contained about 5 times as much nitrogen.

(4) The stability and other properties of our material were different from those of glutathione.

The small activity of glutathione in growth tests would have suggested that this might be due to a biologically active impurity in our glutathione preparations, had not Rane & Subbarow found a synthetic preparation also active.

The somewhat trivial effects suggested that they might be non-specific in the sense already referred to and due merely to an improvement in the general metabolism brought about by increase in the amino-N content of the nutrients. In other work we had found that increases in the concentrations of particular amino-acids might have a critical effect upon growth. We therefore carried out tests in which the constituent amino-acids of glutathione, glycine and glutamic acid, were severally increased in concentration, cystine being already adequately represented.

Table I shows that high concentrations of synthetic glutamic acid were capable of inducing growth comparable in rapidity and mass with that resulting from our meat extract. It was shown that aspartic acid had no such action.

Table I. *Effect of glutamic acid as a replacement for meat extract*

A specimen of *dl*-glutamic acid synthesized by McIlwain & Richardson's [1939] method was dissolved in water, neutralized and autoclaved; concentration *M*/1.5. Dilutions were added to the inadequate mixture to give the concentrations shown.

	Growth after hr.		
	17	24	43
Inadequate medium + O	0	0	0
+ <i>dl</i> -glutamate <i>M</i> /25	++	++++	++++
<i>M</i> /50	?	+	++++
<i>M</i> /100	0	0	++++
<i>M</i> /200	0	0	0
+ meat extract 1.0 ml.	++++	++++	++++
0.2 ml.	+++	+++	+++
0.04 ml.	+	+	++
0.008 ml.	0	0	0

Here and elsewhere, *plus* signs are roughly proportional to the mass of growth.

Our interpretation of the results of Table I was that glutamic acid could not be the active substance in our meat preparations because there were obvious qualitative and quantitative differences between them. Glutamic acid might however be specifically associated with our active substance in the sense that the coccus could synthesize our active substance in adequate amounts in the presence of high concentrations of glutamic acid, but not in adequate amounts when the concentration of glutamic acid was low.

According to this interpretation glutamic acid might be the parent substance from which our active material was synthesized. We therefore surveyed the derivatives of glutamic acid which were known to occur in nature and which had similar properties to those of our active substance and we selected for test glutamine.

It was found that glutamine, sterilized by filtration, when added to the inadequate basal medium in a concentration as low as  $M/5000$  caused full growth in as little as 16–17 hr. This growth was capable of serial subculture in the same mixture as often as desired. Asparagine tested under the same conditions was inactive.

The following specimens of glutamine from different sources had the same activity:

(1) A specimen isolated by Prof. Chibnall from natural sources, containing 98.2 % glutamine (glutamine was determined by the method of Vickery, Pucher Clark *et al.* [1935]).

(2) A specimen prepared by ourselves from beetroot by the method of Vickery, Pucher and Clark [1935], containing 97.5 % glutamine.

(3) A specimen synthesized by Prof. Harington from natural glutamic acid [Harington & Mead, 1935].

(4) A specimen synthesized by ourselves from synthetic glutamic acid.

The results with specimen 4 showed conclusively that glutamine itself and not any associated biologically active impurity was capable of replacing our active meat preparations.

A survey was then made of our various fractions of meat to estimate how far their activities could be ascribed to glutamine in them, determined as labile amide-N by the above method. The results of analysis are given in Table II on

Table II. *Glutamine content and activity of preparations*

	Total N mg./ml.	NH <sub>2</sub> -N mg./ml.	Labile amide-N* mg./ml.	Calc. glutamine %	Activity for growth
Meat extract	27.7	0.37	0.106	0.111	+ + + +
1st Hg ppt.	4.59	0.36	0.105	0.109	+ + + +
Phosphotungstic filtrate	0.672	0.015	0.0403	0.042	+ + +

\* Determined under the conditions of Vickery, Pucher, Clark *et al.* [1935] for glutamine.

solutions equivalent to the original meat extract. Negligible amounts of glutamine were found in the phosphotungstic precipitate and in the less soluble salts separated at that stage (p. 225); the loss of glutamine in the filtrate may readily be explained by the temporary alkalinity and the considerable evaporation involved in this process. It will be seen that the distribution of glutamine-N in the fractions was the same as that of growth activity.

#### *Separation of glutamine from meat*

The method used was essentially that by which Schulze & Bosshard [1883] and Vickery, Pucher & Clark [1935] isolated glutamine from plant extracts. It was not found possible to apply this to the immediate meat extract or first mercury precipitate owing to their containing much smaller amounts of glutamine than a typical beet extract (about 1/20) and larger amounts of associated materials. The method was however successfully applied to the material after phosphotungstic acid precipitation (p. 225) in which the glutamine, with 60 % loss, had been separated from 97.6 % of the associated nitrogenous impurities (Table II).

To material from 1000 ml. of meat extract at this stage, in water (50 ml.), saturated aqueous basic lead acetate solution was added till in excess (c. 40 ml.) while progressively neutralizing the solution with *N* acetic acid (c. 2 ml.). The pale cream-coloured granular precipitate settled quickly and was filtered off after 30 min. and washed 3 times with water (10 ml.). To the mother liquors



a 30% solution of mercuric nitrate [cf. Vickery, Pucher & Clark, 1935] was added in excess (c. 30 ml.). The solution then had pH 2, and the white precipitate (HgP I) was collected and washed after 2 hr. The mother liquors were neutralized with 10*N* NaOH (c. 6 ml.), kept overnight and the further precipitate (HgP II) collected and washed with water. All fractions were decomposed with H<sub>2</sub>S and their growth activities and glutamine contents determined. The material recovered from the lead precipitate had no activity and contained no detectable glutamine; the material from the two mercury precipitates contained the bulk of the glutamine and activity, while the mother liquors were slightly active and contained about 12% of the total glutamine. The solutions containing the bulk of the material recovered from the two mercury precipitates were each neutralized with ammonia, evaporated under reduced pressure from a bath at 50° to about 2 ml. and the separated crystals collected and washed with 50% aqueous alcohol. More crystals were obtained from their mother liquors by treatment with alcohol. The fractions had the properties shown in Table III. The activities of these fractions also were closely correlated with their glutamine contents, and about  $\frac{1}{2}$  the glutamine in the starting material had been isolated.

Table III. *Characters and growth activities of glutamine fractions isolated from meat*

	Wt. g.	M.P.	Mixed M.P. with L-(+) glutamine	Glutamine content		Activity*
				%	g.	
HgPI 1st crop	0.125	194-5°	196-7°	99	0.22	+ + +
HgPI 2nd crop	0.036	194-5°	196-7°			
HgPI 3rd crop	0.087	172-4°	187-9°	69	0.013	+
HgPI mother liq.						
HgPII 1st crop	0.076	>260°		0	0	0
HgPII 2nd crop	0.027	>260°				
HgPII mother liq.					0.120	+ +
Initial phosphotungstic acid precipitated meat extract					0.420	+ + + +

\* Tested in concentration equivalent to that of the original meat extract.

Table IV. *The growth activity of meat in relation to its glutamine content*

Crude meat extract estimated to contain 0.111 mg. glutamine/ml. was titrated against a solution of glutamine (specimen 3) containing 0.111 mg./ml.

				Growth in hr.		
Tube				24	29½	48½
1	Inadequate medium + glutamine	1.0 ml.		+ + + +	+ + + +	+ + + +
2	+ glutamine	0.2 ml.		+ + +	+ + + +	+ + + +
3	+ glutamine	0.04 ml.		+	+ + ⊥	+ + +
4	+ glutamine	0.008 ml.		Tr.	+	+ ⊥
5	+ glutamine	0.0016 ml.		0	Tr.	+
6	+ glutamine	0.00032 ml.		0	0	0
7	+ meat extract	1.0 ml.		+ + + +	+ + + +	+ + + +
8	+ meat extract	0.2 ml.		+ + +	+ + + +	+ + + +
9	+ meat extract	0.04 ml.		+	+ + ⊥	+ + +
10	+ meat extract	0.008 ml.		Tr.	+	+ ⊥
11	+ meat extract	0.0016 ml.		0	0	0
12	+ meat extract	0.00032 ml.		0	0	0
13	+ water	1.0 ml.		0	0	0

The purer glutamine fractions were recrystallized 3 times from aqueous alcohol; the product melted higher (205°) than glutamine previously isolated from natural sources, had a typical optical rotation of  $[\alpha]_D^{20} = +6.5 \pm 0.1^\circ$  and gave the precipitation reactions described in the literature.

In a final experiment it was shown that under the conditions of our test the only contribution made by the meat to the growth of streptococcus was glutamine. A solution of glutamine was prepared at a concentration equivalent to the estimated concentration in our crude meat extract and both were titrated for growth-inducing activity. Table IV shows that the activity of each was approximately the same.

#### DISCUSSION

The use of meat extract as an addition to peptone was, according to Bulloch [1930], introduced by Loeffler in 1881 and has been a routine procedure in medical bacteriology ever since. It would appear that an important function of the meat so far as *Str. haemolyticus* is concerned is to ensure the presence of glutamine in the nutrients. Arguing from the action of animal extracts and fluids (e.g. urine) upon the growth of streptococcus it may be assumed that glutamine is widely distributed in the animal body, but little reference can be found to its possible function. It is however interesting to note that Krebs [1935] has found that the brain cortex and retina of vertebrates and the kidney of the rabbit and guinea-pig can synthesize glutamine from glutamic acid and that extracts of these organs contain a glutaminase which can catalyse the reverse process. It is possible that similar processes can be carried out by streptococci and that they are a necessary feature of their growth.

The effect of glutamine described has also been demonstrated with ten other strains of haemolytic streptococci. Two others grew equally well under the conditions used without glutamine.

#### SUMMARY

This work continues a study of the nutrients essential for the growth of bacteria. Previous work has shown that "simple" non-pathogenic microbes can obtain all nitrogenous compounds required in their metabolism by synthesis from ammonia. Increasing complexity of nutrient requirements, usually associated with increasing pathogenicity, calls for the provision of more complex nutrients some of which have been identified with established animal vitamins. The present paper shows that glutamine, a substance present in animal tissues but with no known function, is an "essential growth factor" for most strains of streptococci isolated from cases of disease in that it must be supplied in the nutrients.

We have pleasure in thanking Prof. A. C. Chibnall and Prof. C. R. Harington for supplying us with specimens of glutamine and Dr L. Colebrook and Dr L. E. H. Whitby for cultures used in the tests.

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# XXVIII. THE RETENTION OF ACIDS BY SERUM BASED ON THE RETENTION COEFFICIENT: ITS USE IN MANOMETRIC EXPERIMENTS

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THE two-vessel method of Warburg [1924] has the unique advantage for the measurement of tissue metabolism that it permits the simultaneous and continuous reading of respiration and total acid production. Whilst serum may be used as the medium for such measurements [Warburg, 1925], and is a more physiological medium than simple saline solutions, the retention of part of the evolved lactic or carbonic acid caused by its interaction with the buffers other than bicarbonate brings with it the need for special retention corrections.

Hitherto, two procedures have been available: (1) In the original method of Warburg [1925] the retentions for lactic and carbonic acids are calculated from the data of a separate acidification experiment, and from the bicarbonate and  $\text{CO}_2$  contents of the serum. The correction obtained applies only to experiments in which the change of  $\text{CO}_2$  pressure is close to that found in the acidification experiment. (2) Some of the inconvenience of the former method is avoided by acidification in the actual vessels used for measurement of tissue metabolism [Warburg *et al.* 1931], all of which must therefore be provided with side-bulbs. This is not always convenient, and some loss of accuracy may result from the fact that the acidification is made with the tissue present in the vessels. With some tissues the addition of lactic acid may cause an appreciable change in metabolism. Even slight errors in reading may cause an appreciable error in the retention determination, and it is not easy to secure high accuracy in presence of respiring or glycolysing tissue. In this method also, the retention correction applies only to a pressure change close to that found in the acidification experiment. Separate retention estimations for  $\text{CO}_2$  and lactic acid are necessary.

In the principle to be described, instead of basing the calculation of retention on the Michaelis dissociation residue, as was done by Warburg [1925], we have worked from the empirical finding that the retention, or decrease of alkali proteinate, is directly proportional to the change of  $p\text{H}$ . This was first shown by Van Slyke *et al.* [1923] for dialysed serum, and afterwards by Dickens & Šimer [1932] and Dixon [1937] for normal serum. Over the physiological range of  $p\text{H}$  the relationship may be regarded as linear, and an essential simplification is thus introduced. It is also possible with this method to calculate the retention for any observed pressure change.

## Nomenclature

The following symbols are used throughout:

$\text{H}^+$  = hydrogen ion concentration of the serum.

$K$  = 1st dissociation constant of carbonic acid (for serum we take  $K = 7.94 \times 10^{-7}$ ,  $pK = 6.10$  [Hastings *et al.* 1928]).

$p$  = partial pressure of  $\text{CO}_2$  (mm. Brodie solution).

$B$  = bicarbonate concentration ( $\mu\text{l}$ . per ml.).

$\beta$  = solubility coefficient of  $\text{CO}_2$  in serum ( $\mu\text{l. CO}_2/\text{ml.}$  for a pressure of 1 atm. see below).

$v_F$  = volume of serum in vessel (ml.).

$\Delta u$  = retention of  $\text{CO}_2$  per ml. serum per mm. change of  $p$  (where  $u$  is the hypothetical concentration of the acidic buffers other than bicarbonate, e.g. proteinates, in the serum, expressed in  $\mu\text{l./ml.}$  as usual).

All pressures are reckoned in mm. Brodie fluid, of which 10,000 mm. = 1 atm. Volumes of gas, or of acids and bases considered as gases in the usual convention, are in  $\mu\text{l.}$  at N.T.P. Volumes of liquids are in ml. throughout.

With these units, the solubility coefficient,  $\beta$ , is equal to  $\alpha/10$ , where  $\alpha$  is the Bunsen absorption coefficient of the gas. For values of  $\alpha$  for various salt solutions and for serum see Van Slyke *et al.* [1928].

### The "Retention Coefficient" $R$

Since within the limits shown by Dickens & Šimer [1932] the retention- $p\text{H}$  curve of normal serum is linear, we may write

$$-\frac{du}{d(p\text{H})} = R \quad \dots\dots(1),$$

where  $R$  is an empirical constant which will be called the retention coefficient, denoting the retention by 1 ml. serum, expressed in  $\mu\text{l.}$  per unit change of  $p\text{H}$ .  $R$  is a positive quantity, and its value varies between about 100 and 200 for normal sera of different species.

$$\text{Now} \quad R = -\frac{du}{d(p\text{H})} = -\frac{du}{d\text{H}^+} \frac{d\text{H}^+}{d(-\log_{10} \text{H}^+)} = 2.303 \frac{du}{d\text{H}^+} \text{H}^+ \quad \dots\dots(2).$$

It may be shown (Table I) that, to a first approximation, taking finite increases and the mean value of  $\text{H}^+$  over the interval  $\text{H}_0^+$  to  $(\text{H}_0^+ + \Delta\text{H}^+)$

$$R = 2.303 \frac{\Delta u}{\Delta\text{H}^+} \left( \text{H}_0^+ + \frac{\Delta\text{H}^+}{2} \right) \quad \dots\dots(2a),$$

$$\text{whence} \quad \frac{\Delta\text{H}^+}{\text{H}_0^+} = \frac{2.303\Delta u}{R - \frac{2.303\Delta u}{2}} \quad \dots\dots(3).$$

Table I. Accuracy of equation (3)

From equation (1)  $\Delta u/R = -\Delta p\text{H}$ . From equation (2a)  $\Delta u/R \sim \Delta\text{H}^+/2.303 \left( \text{H}_0^+ + \frac{\Delta\text{H}^+}{2} \right)$ .

In Table I these two values of  $\Delta u/R$  are compared. The difference is the error in equation 2a.

$p\text{H}$	$\text{H}^+ \times 10^8$	$\Delta\text{H} \times 10^8$	$\Delta u/R$		Difference %
			$\frac{\Delta\text{H}^+}{2.3 \left( \text{H}_0^+ + \frac{\Delta\text{H}^+}{2} \right)}$	$-\Delta p\text{H}$	
7.3979	4 (= $\text{H}_0$ )	—	—	—	—
7.3468	4.5	0.5	0.0510	0.0511	0.2
7.3010	5	1	0.0965	0.0969	0.4
7.2596	5.5	1.5	0.1369	0.1383	1.0
7.2218	6	2	0.1737	0.1761	1.4

### Acidification of serum

1. *General case.* The partial pressure of  $\text{CO}_2$  in equilibrium with the serum increases from  $p_0$  to  $p_0 + \Delta p$ . Since

$$p_0 = B_0 \text{H}_0^+ / K\beta \quad \text{and} \quad p_0 + \Delta p = (B_0 + \Delta B) (\text{H}_0^+ + \Delta\text{H}^+) / K\beta$$

therefore

$$\frac{\Delta p}{p_0} - \frac{\Delta B}{B_0} = \frac{\Delta\text{H}^+}{\text{H}_0^+} \left( 1 + \frac{\Delta B}{B_0} \right) \quad \dots\dots(4).$$

Substituting from equation (3) the value of  $\Delta H^+ / H_0^+$

$$\frac{\Delta u}{\Delta p} = \frac{R \left( 1 - \frac{p_0 \Delta B}{B_0 \Delta p} \right)}{2.303 p_0 \left( 1 + \frac{\Delta B}{2B_0} + \frac{\Delta p}{2p_0} \right)} \quad \dots\dots(5).$$

Equation (5) represents the general case when  $p$ ,  $B$  and  $u$  vary. Two special cases are important.

(a) *Acidification of serum by addition of lactic or other strong acid.* In a vessel of total volume  $v$  ml., containing  $v_F$  ml. serum and  $v_T$  ml. total liquid including serum, maintained at a temperature of  $T^\circ$  absolute, let  $m$   $\mu$ l. lactic acid be added to the serum, and the resulting increase of partial pressure of  $\text{CO}_2$  be  $\Delta p$  mm. Brodie.

The "vessel constant for Ringer solution"  $k_{v_0}^R$  [Warburg, 1925] is given by

$$k_{v_0}^R = \frac{273}{10T} (v - v_T) + v_T \beta.$$

Change of bicarbonate concentration on acidification  $= \Delta B$

$$\Delta B = -\Delta p \ k_{v_0}^R / v_F.$$

Substituting in (5) and writing the quotient with suffix  $L$ , denoting acidification by lactic acid

$$\left( \frac{\Delta u}{\Delta p} \right)_L = \frac{R}{2.303} \frac{1 + \frac{p_0 k_{v_0}^R}{B_0 v_F}}{p_0 + \frac{\Delta p}{2} \left( 1 - \frac{p_0 k_{v_0}^R}{B_0 v_F} \right)} \quad \dots\dots(6).$$

Equation (6) shows that the retention for lactic acid is dependent on the vessel constant and volume of serum. In these respects it differs from the retention for  $\text{CO}_2$ . The term in  $\Delta p$  indicates the extent of variation of the retention with  $\text{CO}_2$  pressure-change, which was not previously calculable.

(b) *Acidification of serum by addition of carbonic acid.* Since for each equiv. of  $\text{CO}_2$  which is combined, 1 equiv. of  $\text{HCO}_3^-$  results, accompanied by an increase of 1 equiv. in  $u$ , in this case

$$\Delta u = \Delta B.$$

Substituting in (5), and writing the quotient with suffix  $C$ , denoting retention for  $\text{CO}_2$

$$\left( \frac{\Delta u}{\Delta p} \right)_C = \frac{R}{2.303 \left\{ p_0 + \frac{\Delta p}{2} \left( 1 + \frac{p_0 \Delta u}{B_0 \Delta p} \right) \right\} + \frac{R p_0}{B_0}} \quad \dots\dots(7).$$

Equation (7) shows that the retention for  $\text{CO}_2$  is independent of the vessel constant and the volume of serum, but depends on the particular value of  $\Delta p$  as is evident from the bracketed term in the denominator. This term, involving  $(\Delta u / \Delta p)$  is nearly equal to  $p_0 + \frac{\Delta p}{2}$  unless  $p_0$  is large compared with  $B_0$ , since the average value of  $(\Delta u / \Delta p)$  is of the order 0.1. Consequently we may first calculate an approximate value of  $(\Delta u / \Delta p)$  using the approximate relationship

$$\left( \frac{\Delta u}{\Delta p} \right)_C \sim \frac{R}{2.303 \left( p_0 + \frac{\Delta p}{2} \right) + \frac{R p_0}{B_0}} \quad \dots\dots(7a),$$

and afterwards insert this value of  $(\Delta u / \Delta p)$  in the denominator of equation (7). In many cases the effect of this correction term will be almost negligible, but the calculation is very easy in practice, so that it is better to insert the correction as a routine.

*Determination of the retention coefficient, R*

Since  $R$  represents the retention per unit change of  $pH$ , it may be determined by any of the three methods available for determining retention [Warburg, 1925]. These are (1) acidification with a known amount of lactic acid; (2) acidification with a known amount of  $CO_2$  and (3) dilution of the serum. The third method is complicated by the fact that since  $R$  is dependent on the concentration of non-bicarbonate buffers, the value of  $R$  will change during dilution. For this reason methods (1) and (2) are preferable.

The calculation of  $R$  may also be made by either of two methods: (a)  $R$  may be derived from the above equations (6) and (7) for  $(\Delta u/\Delta p)$  or (b)  $R$  may be calculated directly from the data of the acidification experiment, using a logarithmic method. Since these calculations afford an excellent check on the accuracy of the various expressions, they will be described separately.

1a. *Calculation of R by acidification with lactic acid: derivation from  $(\Delta u/\Delta p)_L$ .* Let  $\epsilon$  ml. of a solution of lactic acid containing  $m$   $\mu$ l. acid be added to  $v_F$  ml. serum contained in a vessel of Ringer constant  $k_{CO_2}^R$ , and let the observed pressure change be  $h$  mm. Brodie. Let the pressure change due to dilution of the serum thus caused be  $h'$  mm.; it should be noted that  $h'$  is negative [Warburg, 1925] and its sign must be taken algebraically. Let  $H$  be the pressure change due to acidification alone, without dilution.

Then

$$H = h - h',$$

$$\Delta u = \frac{m - Hk_{CO_2}^R}{v_F},$$

$$\Delta p = h.$$

Hence  $(\Delta u/\Delta p)_L$  is directly calculated from the acidification experiment. If now  $\epsilon$  is a small volume, e.g.  $< 5\%$  of  $v_F$ ,  $R$  will remain sensibly constant during dilution. Hence from equation (6)

$$R = \frac{2.303 (m - Hk_{CO_2}^R) \left( p_0 + \frac{h}{2} - \frac{h p_0 k_{CO_2}^R}{2 v_F B_0} \right)}{h \left( v_F + \frac{p_0 k_{CO_2}^R}{B_0} \right)} \quad \text{.....(8).}$$

1b. *Calculation of R from acidification with lactic acid: logarithmic method.* In the above experiment, the initial conc. of bicarbonate =  $B_0$ . Final conc. of bicarbonate after acidification

$$= B_1 = \frac{B_0 v_F - h k_{CO_2}^R}{v_F + \epsilon}.$$

Hence

$$\Delta pH = pH_1 - pH_0 = \log \frac{B_1}{(p_0 + h) \beta} - \log \frac{B_0}{p_0 \beta}$$

and

$$\Delta u = (m - h k_{CO_2}^R) / v_F.$$

Therefore

$$R = - \frac{\Delta u}{\Delta pH} = + \frac{m - h k_{CO_2}^R}{v_F} \bigg/ \log \frac{\left(1 + \frac{\epsilon}{v_F}\right) \left(1 + \frac{h}{p_0}\right)}{1 - \frac{h k_{CO_2}^R}{B_0 v_F}} \quad \text{.....(9).}$$

This equation eliminates the need for a dilution correction, and is the best form to use.

2a. *Calculation of R from acidification with  $CO_2$ : logarithmic method.* In this case the acidification occurs in a special vessel of the type described by Warburg *et al.* [1931]. An outer compartment contains  $NaHCO_3$ - $NaCl$  solution, into which  $m$   $\mu$ l. lactic acid are tipped, thus liberating  $m$   $\mu$ l.  $CO_2$ . In an inner compartment of the same vessel are  $v_F$  ml. serum. Part of the  $CO_2$  liberated from the

$\text{NaHCO}_3$  recombines with the buffers of the serum. The pressure change observed is  $h$  mm.

Then

$$\Delta u = (m - h k_{\text{CO}_2}^R) / v_F,$$

$$\Delta p\text{H} = \log \left( 1 + \frac{m - h k_{\text{CO}_2}^R}{B_0 v_F} \right) \left( \frac{p_0}{p_0 + h} \right) \quad \text{.....(10).}$$

Whence  $R = -(\Delta u / \Delta p\text{H})$  is readily calculated.

2b. *Calculation from equation (7).* In the above experiment,  $\Delta u$  is given and  $h = \Delta p$ . Hence the quotient  $(\Delta u / \Delta p)_0$  is directly obtained and from (7):

$$R = \frac{2.3 (\Delta u / \Delta p)}{1 - \frac{p_0 \Delta u}{B_0 \Delta p}} \left\{ p_0 + \frac{\Delta p}{2} \left( 1 + \frac{p_0 \Delta u}{B_0 \Delta p} \right) \right\} \quad \text{.....(11).}$$

#### *Vessel constants*

When the values of  $(\Delta u / \Delta p)_C$  and  $(\Delta u / \Delta p)_L$  for the required range of  $\Delta p$  have been calculated by means of equations (6) and (7), it is only necessary to insert these values in equations (12) and (13) to obtain the respective vessel constants for serum [Warburg, 1925].

$$k_{\text{CO}_2}^S = k_{\text{CO}_2}^R + v_F \left( \frac{\Delta u}{\Delta p} \right)_C \quad \text{.....(12),}$$

and

$$k_L^S = k_{\text{CO}_2}^R + v_F \left( \frac{\Delta u}{\Delta p} \right)_L \quad \text{.....(13).}$$

The calculation of the result of a 2-vessel metabolic experiment may then be made either by the original Warburg [1925] method, or by the method of Warburg *et al.* [1931]. Average values of R.Q. for various tissues, which are required for the latter method, are given by Dickens & Šimer [1930; 1931, 2].

#### *Retention in the Dickens-Šimer R.Q. method*

In the direct method for measurement of metabolism in bicarbonate media [Dickens & Šimer, 1931; 1932; Dixon & Keilin, 1933; Dixon, 1937] the measurement of respiration is not affected by retention, unlike the same measurement by the Warburg 2-vessel method. But for the measurement of glycolysis by these methods the present principle has the advantage of greater accuracy compared with the "retention curve" estimations previously described [Dickens & Šimer, 1932; Brekke & Dixon, 1937].  $\Delta p\text{H}$  being known directly from the manometric readings [cf. Dickens & Šimer, 1932; 1933] it is only necessary to multiply this by  $R$  in order to obtain the actual retention, in  $\mu\text{l.}$  per ml. serum, which occurred during the experiment.  $R$  is determined as usual by acidification by lactic acid. Only two extra vessels (with Brodie fluid in the manometers) are needed, one for acidification of serum and one for estimation of the strength of the lactic acid (cf. Table IV, vessels 2 and 4).

#### *Dilution correction*

When  $R$  is determined from the value of  $(\Delta u / \Delta p)_L$  (but not by the logarithmic method) a correction  $h'$  due to the change of  $p\text{H}$  of the serum on dilution with the small volume  $\epsilon$  has to be applied (equation 8). The separate determination of  $h'$  is generally unnecessary, since it may be readily calculated from the value of  $R$ , as follows (the suffix  $D$  denotes dilution):

$$\Delta \text{H}_{D^+} = \frac{\beta K (p_0 + h')}{B_0 \frac{v_F}{v_F + c} - \frac{h' k_{\text{CO}_2}^R}{v_F + c}} - \frac{\beta K p_0}{B_0} \quad \text{.....(14).}$$

If  $h'$  is small (it is c.  $-5$  mm. for  $\epsilon=0.1$  ml.) and  $B_0$  and  $k_{CO_2}^R$  are of the usual order (c. 500 and 1 respectively),  $h'k_{CO_2}^R$  is negligible in relation to  $B_0v_F$  and since  $p_0$  is usually c. 400,  $(p_0+h') \sim p_0$

$$\Delta H_{D^+} = \frac{\beta K p_0 (\epsilon)}{B_0} = H_0 + \frac{\epsilon}{v_F}$$

and

$$\Delta u = -\frac{h'k_{CO_2}^R}{v_F}$$

Hence from equation (2), regarding  $R$  as constant for this slight dilution,

$$h' = -\frac{R\epsilon}{2.3k_{CO_2}^R} \quad \dots\dots(15).$$

For the purpose of equation (15),  $R$  may be given an approximate value of 150, provided that the volume  $\epsilon$  does not exceed 0.1 ml. The error in  $h'$  is then unlikely to exceed c. 1.5 mm. in an extreme case. If greater accuracy is desired the logarithmic method of calculation (equation 9) may be used. The dilution effect may be avoided completely by using evaporated tartaric acid solution instead of lactic acid [Warburg, 1925] and writing  $\epsilon=0$  and  $h'=0$  in the above equations (8) and (9), but the use of a solution is convenient.

#### Typical case

For an average specimen of normal serum at  $37.5^\circ$  the following values are typical:  $R=150$ ,  $p_0=420$ ,  $B_0=427$ ,  $\alpha=0.51$  [Van Slyke *et al.* 1928], or  $\beta=0.051$ ,  $p_0\beta=21.4$ ,  $pK=6.10$  or  $K=7.94 \times 10^{-7}$  [Hastings *et al.* 1928],  $pH_0=7.40$  or  $H_0^+=4 \times 10^{-8}$ .

Table II shows values of  $\Delta u/\Delta p$  for this specimen of serum. The values of  $(\Delta u/\Delta p)_C$  are calculated from equation (7). Correction of the value (e.g. for  $\Delta p=100$ ) by means of equation (7a) only changes the value from 0.122 (uncorr.)

Table II. Calculation of  $(\Delta u/\Delta p)$  and  $\Delta pH$  for a typical specimen of serum

For details see text. Lactic retention calc. for a vessel of total volume 13.2 ml.; for  $v_F=2$  ml.,  $k_{CO_2}^R=1.09$ ; for  $v_F=7$  ml.,  $k_{CO_2}^R=0.902$ .  $R=150$ .

#### Retention for $CO_2$

$\Delta p$ mm. Brodie	$(\Delta u/\Delta p)_C$ from equation (7)	$\Delta u$ $=\Delta p(\Delta u/\Delta p)_C$	$\Delta pH$ $=-\Delta u/R$
0	0.135	0	0
50	0.126	6.3	-0.042
100	0.119	11.9	-0.079
150	0.112	16.8	-0.112
200	0.106	21.2	-0.141

#### Retention for lactic acid; $(\Delta u/\Delta p)_L$ calc. from equation (8)

$\Delta p$ mm. Brodie	For $v_F=2$			For $v_F=7$		
	$(\Delta u/\Delta p)_L$	$\Delta u$	$\Delta pH$	$(\Delta u/\Delta p)_L$	$\Delta u$	$\Delta pH$
0	0.240	0	0	0.175	0	0
50	0.234	11.7	-0.078	0.166	8.3	-0.055
100	0.228	22.8	-0.152	0.158	15.8	-0.105
150	0.222	33.3	-0.222	0.151	22.6	-0.151
200	0.216	43.2	-0.288	0.145	29.0	-0.193

to 0.119 (corr.), an amount which is almost negligible in practice. On the other hand the effect of the correction for various values of  $\Delta p$  is quite large, and this explains why with the Warburg [1925] method of calculation, the retention could only be determined for one particular experimental value of  $\Delta p$ .



The values of  $(\Delta u/\Delta p)_L$  in Table II are calculated from equation (6) for the same specimen of serum. For the same vessel, two series of values are given to show the effect of varying  $v_F$ . The effect of  $\Delta p$  resembles that found for retention of  $\text{CO}_2$  (Table II).

For a vessel of  $k_{\text{CO}_2}^*$  about 1.0, an increase of  $p$  of about 100 mm. Brodie causes a decrease of the vessel constant for serum of about 5%. The effect of this in an experiment by the 2-vessel method may be to alter the metabolic quotient by an amount which may reach 10% of its value.

The change in  $p\text{H}$  corresponding to any value of  $\Delta p$  may be readily computed from the equation:  $\Delta p\text{H} = -\Delta p (\Delta u/\Delta p)/R$ , in which it is important to use the correct quotient and value of  $\Delta p$  for  $\text{CO}_2$  or lactic acid respectively. The values of  $\Delta p\text{H}$  included in Table II show the order to be expected. This calculation is useful for two purposes; it enables one to guard against too great an acidity of the medium, and it may also be used in conjunction with Table I as a check on the error introduced by the approximation in equation (2a).

#### *Practical details*

*Serum.* The serum used in these experiments was obtained by allowing the blood to clot, centrifuging the supernatant serum, and inactivating by heating for two successive periods of 2 hr. at  $56^\circ$ .

The serum was warmed to  $37^\circ$  and shaken with the gas-mixture before use. It is advisable, and with larger volumes of serum essential, to pass the gas stream through the vessels containing the serum, while they are shaking in the bath at  $37^\circ$ . A comparison of the retention of 7 ml. serum contained in a vessel of total vol. 14 ml. gave, by previous gassing in the laboratory and then transferring to the bath  $(\Delta u/\Delta p)_M = 0.131$ ; by gassing while shaking in the bath  $(\Delta u/\Delta p)_M = 0.193$ . Consequently the vessels should be provided with bored-out stoppers, and the gassing arrangement recommended by Dickens & Šimer [1933, p. 450] should be used.

*Lactic acid solution.* The solution, *c.*  $M/20$ , is refluxed for about 1 hr., cooled,  $\text{NaCl}$  added to make the conc. 0.9% and the strength of the solution determined manometrically. The strength remains unchanged for many weeks if the solution is kept sterile by occasionally heating in the stoppered flask in a boiling water bath.

Accurate measurement of the solution into the side-bulbs by a good, grease-free micro-pipette is essential.

The details of a complete retention estimation are given in Table III. The gas analysis is by the method described by Dickens & Šimer [1931, 1], using Clerici solution of density *c.* 4 as manometric fluid. If a Brodie thermobarometer is used, its readings must be divided by 4 before correcting the pressures in the gas analysis. Naturally the gas analysis is needed only when a new cylinder of gas is employed; correction of  $p_0$  for various temperatures and barometric pressures may easily be made, if necessary.

In general, the acidification by lactic acid is to be preferred to the direct determination of  $(\Delta u/\Delta p)_C$  as in vessel 1, Table III. Owing to the larger vessel volume necessary, the latter method is less accurate and cannot easily be applied to larger volumes of serum. An example is shown in Table III, for which the bicarbonate-R.Q. vessel of Dickens & Greville [1933] was used, and proved quite suitable; but it will be seen that the value of  $\Delta u$  obtained is greatly affected by a very small error in  $h$ . Consequently it is better to calculate  $(\Delta u/\Delta p)_C$  from an experiment of the type shown in vessel 2. If the volume of serum is limited, 2 or 3 ml. may be used, or less with smaller vessels. The latter

Table III. *Protocol of a complete retention estimation*

Pig serum. 37.5°. O<sub>2</sub> + CO<sub>2</sub> mixture passed through all vessels for 8 min. while shaking in the bath.  $\alpha_{\text{CO}_2}$  for serum = 0.51, for Ringer solution = 0.54. Manometer fluid: Brodie solution, except No. 5 which was Clerici fluid of density 3.717. Strength of lactic acid solution: *c.* N/20 in 0.9% NaCl. Vessels: No. 1 bicarbonate-R.Q. type [Dickens & Greville, 1933]; Nos. 2 and 4 rectangular with side-bulb; Nos. 3 and 5 conical with side-bulb. All vessels with bored-out stoppers for gassing in bath. Barometer 758.2 mm. Hg.

Vessel no. ...	1	2	3	4	5
Exp.	CO <sub>2</sub> retention	Lactic retention	Bicarbonate estimation	Strength of lactic acid	Gas analysis
Vessel vol. (ml.)	23.72	13.52	16.36	14.56	<i>c.</i> 20
$v_T$	4.10	7.10	0.70	2.1	2.3
$v_F$ (serum)	2	7	0.5	—	—
$k_{\text{CO}_2}^R$	1.943	0.926	1.411	1.206	—
Vessel contents:					
Main pt.	2 ml. serum	7 ml. serum	0.5 ml. serum	2 ml. NaHCO <sub>3</sub> -Ringer	2 ml. M/5 KMnO <sub>4</sub> in N/500 H <sub>2</sub> SO <sub>4</sub>
Outer ring	2 ml. NaHCO <sub>3</sub> -Ringer	—	—	—	—
Side-bulb	0.1 ml. lactic acid	0.1 ml. lactic acid	0.2 ml. N HCl	0.1 ml. lactic acid	0.3 ml. 30% NaI, 2H <sub>2</sub> O in N/500 H <sub>2</sub> SO <sub>4</sub>
Pressure change on mixing (corrected for thermobarometer vessel):					
$h$	+ 49.5	+ 46.5	+ 155.5	+ 92 (Brodie)	* - 81 (Clerici)
	$hk_{\text{CO}_2}^R + 96$	+ 43	$2hk_{\text{CO}_2}^R - B_0 = 439$	$hk_{\text{CO}_2}^R = m - 111$	$p_0 = 81 \times \frac{3.717}{13.60} \times \frac{10,000}{760} = 290 \text{ mm. Brodie}$
Dilution corr.	$h' = 0$	$h' = -5$	—	—	—
$(h - h') k_{\text{CO}_2}^R$	+ 96	+ 48	—	—	—
$(m - Hk_{\text{CO}_2}^R)/v_F = \Delta u$ :	7.5	9.0	—	—	—
$\Delta u/h$	$(\Delta u/\Delta p)_C = 0.151$ for $\Delta p \sim 50$	$(\Delta u/\Delta p)_L = 0.193$ for $\Delta p \sim 45$	—	—	—
Calculation according to Warburg [1925]:					
	$(\Delta u/\Delta p)_C = 0.153$ for $\Delta p_C = 59$	$(\Delta u/\Delta p)_L = 0.192$ for $\Delta p_L = 46$	—	—	—
Calculation of $R$ :					
(a) From equations (8) and (11)	$R = 123$	$R = 127$	—	—	—
(b) From equations (9) and (10)	$R = 123$	$R = 127$	—	—	—
Calculation of $(\Delta u/\Delta p)$ , for mean value of $R = 127$ , from equations (6) and (7), for above values of $\Delta p$ :					
	$(\Delta u/\Delta p)_C = 0.152$	$(\Delta u/\Delta p)_L = 0.193$	—	—	—

\* Corrected for pressure change of - 3 mm. on mixing reagents [Dickens & Šimer, 1931, 1].

must be carefully made to provide a large surface of the serum, or equilibration with the gas mixture is slow. When using small volumes of serum care must be taken that the dilution effect is small (equation 15), and it may therefore be necessary to use evaporated tartaric acid instead of lactic acid.

Since the gas analysis will generally be done at once when the gas mixture is received, only three vessels are necessary for a complete retention estimation, viz. nos. 2, 3 and 4, Table IV. From the values of  $h$ ,  $B_0$  and  $m$  thus obtained, the retentions for lactic and carbonic acids for any desired values of  $\Delta p$  may at once be calculated by means of equations (9), (6) and (7). The value of  $R$  obtained

from equation (9) may be used to calculate the retention in any experiment in which the same specimen of serum is used at a value of  $pH$  within the physiological range.

*Relation of retention to protein content of serum*

According to Van Slyke *et al.* [1923] for dialysed horse serum the empirical relationship

$$\frac{BP}{(pH - 4.80)} = 0.68P$$

holds for the physiological range, where  $BP$  is the millimolar conc. of base bound by protein, and  $P$  the total protein in g./100 ml. Hence in our units for  $u$

$$\Delta u = -\Delta (BP) \times 22.4,$$

and

$$R = -du/d(pH) = 0.68 \times 22.4P = 15.2P.$$

Table IV shows the relationship of  $R$  to  $P$  for 6 specimens of (undialysed) normal serum. The variation of  $R$  from  $15P$  to  $22P$  is rather large, not only with different species, but also with different sera of the same species. Hence it is not practicable to calculate  $R$  from the protein content of the serum, but is better to determine it manometrically for each specimen. The coincidence of the retention- $pH$  curves for two particular specimens of horse-serum [Dickens & Šimer, 1932] and sheep-serum [Dixon, 1937] must therefore be regarded as fortuitous.

Table IV. *Value of R and total protein in various specimens of serum*

Species	...	Pig	Calf	Sheep I	Sheep II	Horse I	Horse II
Mean $R^*$		131	108	183	108	106	107
Total protein %		7.20	4.98	6.25	6.42	7.10	6.35
$R/(\%$ protein)		18.2	21.6	22.2	16.8	15.0	16.9

\* Mean of 7 estimations.

*Retention by substances of known  $pK'$*

In manometric experiments made in simple  $\text{NaHCO}_3$ -saline media, it is often necessary to consider if the retention of added substrates or other substances need be taken into account. Table V shows the retentions due to substances of various  $pK'$ , for different reactions of the medium.

Table V. *Value of R for various values of  $K'$*

For a substance of dissociation constant  $K'$ , dissolved in conc.  $c'$  ( $\mu\text{l./ml.}$ ) in a bicarbonate medium in equilibrium with  $\text{CO}_2$  [Dickens & Šimer, 1932]

$$R = -\frac{dp}{d(pH)} c' = 2.3 \frac{H^+ K'}{(H^+ + K')^2} c'.$$

The following Table shows values of  $R$  for various values of  $H^+$  and  $K'$ : the value of  $c'$  taken is equivalent to  $M/130$  and gives  $R_{\max} = 100$ .

$pK'$	$R$ for $c' = 174$ at		
	$pH = 6.0$	$pH = 7.0$	$pH = 8.0$
4	4	0.4	0.04
5	33	4	0.4
6	100	33	4
7	33	100	33
8	4	33	100
9	0.4	4	33
10	0.04	0.4	4

Hence for a change of 0.1 pH unit the retention by  $M/100$  buffer will be less than 0.5  $\mu$ l. per ml. solution, if the  $pK'$  of the buffer differs from the pH of the medium by more than 2. For other values of  $pK'$  and pH the retention can be calculated from the above equation. It should be noted that the effect of temperature and salt concentration on  $pK'$  may cause a considerable alteration in retention.

#### SUMMARY

The retention of acids by serum has been considered in relation to the change of pH of the serum. The "Retention Coefficient"  $R$ , representing the amount of retention per unit decrease of pH, has been used to calculate the retention of carbonic and lactic acids by serum. Methods are described for the calculation and estimation of  $R$ .

The application of this principle to the measurement of tissue metabolism in serum is described. By this means it is possible to calculate the value of the retention for any given pressure change, an advantage not possessed by other methods of determining retention.

The retention caused by the presence of substances of known dissociation constant is described.

I am grateful to Dr F. J. W. Roughton and Dr M. Dixon for useful criticism.

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## XXIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

### LX. GRISEOFULVIN, $C_{17}H_{17}O_6Cl$ , A METABOLIC PRODUCT OF *PENICILLIUM GRISEO-FULVUM* DIERCKX

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THE following metabolic products, present in the metabolism solution of *Penicillium griseo-fulvum* Dierckx when this mould is grown under different cultural conditions and on different culture solutions, have been isolated in this laboratory: 6-hydroxy-2-methylbenzoic acid [Anslow & Raistrick, 1931], 2:5-dihydroxybenzoic acid (gentisic acid), fumaric acid and mannitol [Raistrick & Simonart, 1933] and fulvic acid,  $C_{14}H_{12}O_8$ , a yellow crystalline substance of at present undetermined molecular constitution [Oxford *et al.* 1935]. The purpose of the present communication is to record observations on a hitherto undescribed chlorine-containing metabolic product of *P. griseo-fulvum* which has been isolated from the mycelium of this mould and for which the name *griseofulvin* is proposed.

Griseofulvin,  $C_{17}H_{17}O_6Cl$ , M.P. 218–219°, is a colourless, crystalline, neutral compound giving no colour with  $FeCl_3$  and containing no free hydroxyl or carboxyl groups. It is highly dextrorotatory,  $[\alpha]_{5461} + 417^\circ$ .

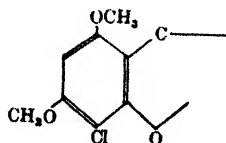
One oxygen atom is present as  $>CO$  since a crystalline mono-oxime,  $C_{17}H_{16}O_6NCl$ , was readily obtained; derivatives are also formed with phenylhydrazine and with 2:4-dinitrophenylhydrazine, but these could not be obtained crystalline. The function of three other oxygen atoms is obvious since griseofulvin contains three methoxyl groups. A study of the products obtained on acid and alkaline hydrolysis showed that griseofulvin must be the methyl ester of a carboxylic acid, and hence the function of a fifth oxygen atom is accounted for. The function of the sixth oxygen atom has not been definitely settled though evidence given later indicates that it is present as an oxygen bridge.

Griseofulvin, when hydrolysed with boiling *N* aqueous-alcoholic  $H_2SO_4$ , yields *griseofulvic acid*,  $C_{16}H_{15}O_6Cl$ ,  $[\alpha]_{5461} + 508^\circ$ , a monobasic acid containing two methoxyl groups and giving only a feeble colour with  $FeCl_3$ , but these facts in themselves are not sufficient to establish the presence of the grouping  $-COO.CH_3$ . However, hydrolysis of griseofulvin, or further hydrolysis of griseofulvic acid, with boiling aqueous *N*/2  $NaOH$  yields *norgriseofulvic acid*,  $C_{15}H_{13}O_6Cl$ ,  $[\alpha]_{5461} + 609^\circ$ , a dibasic acid containing only one methoxyl group, together with *decarboxygriseofulvic acid*,  $C_{15}H_{15}O_4Cl$ ,  $[\alpha]_{5461} - 31^\circ$ , an insoluble neutral compound containing two methoxyl groups, giving no colour with  $FeCl_3$ , and derived from griseofulvic acid by the loss of 1 mol. of  $CO_2$ . Decarboxy-griseofulvic acid is stable to acid hydrolysis and hence it seems certain that griseofulvin contains only one  $-COO.CH_3$  group and that the second acidic

group in *norgriseofulvic* acid is a phenolic and not a carboxyl group. This conclusion is supported by the colour reactions of *norgriseofulvic* acid, an intense brown colour with alcoholic  $\text{FeCl}_3$ , an intense orange brown colour with diazotized sulphanilic acid in  $\text{Na}_2\text{CO}_3$  solution and a positive Millon reaction.

On catalytic reduction with palladium-charcoal-hydrogen, griseofulvin gives rise to two reduction products: *dihydrogriseofulvin*,  $\text{C}_{17}\text{H}_{19}\text{O}_6\text{Cl}$ ,  $[\alpha]_{5461} - 33'$ , and *tetrahydrodeoxygriseofulvin*,  $\text{C}_{17}\text{H}_{21}\text{O}_5\text{Cl}$ . Dihydrogriseofulvin still contains the  $\text{>CO}$  group present in griseofulvin since it forms a 2:4-dinitrophenylhydrazone with Brady's reagent. Hence the two hydrogen atoms taken up in the formation of dihydrogriseofulvin must have been absorbed in the saturation of a  $\text{—C=C—}$  linkage. This conclusion is supported by the fact that whereas griseofulvin gives no coloration with Tüfel & Thaler's [1932] reagent—warming with salicylaldehyde and 45 % aqueous  $\text{H}_2\text{SO}_4$ —dihydrogriseofulvin gives a deep red colour with this reagent. This indicates clearly the presence in dihydrogriseofulvin of the grouping  $\text{—CH}_2\text{.CO.CH}_2\text{—}$  so that griseofulvin itself must contain the grouping  $\text{—CH}_2\text{.CO.CH=}$ . Tetrahydrodeoxygriseofulvin contains two hydrogen atoms more and one oxygen atom less than dihydrogriseofulvin. This fact is most readily explained by assuming that the grouping  $\text{—CH}_2\text{.CO.CH}_2\text{—}$  in dihydrogriseofulvin becomes  $\text{—CH}_2\text{.(CH}_2\text{.CH}_2\text{—)}$  in tetrahydrodeoxygriseofulvin, an assumption that is supported by the fact that tetrahydrodeoxygriseofulvin does not react with Brady's reagent.

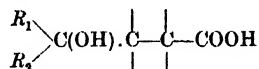
On oxidation of griseofulvin with  $\text{KMnO}_4$  in acetone at room temperature two degradation products were isolated. The first of these,  $\text{C}_9\text{H}_9\text{O}_5\text{Cl}$ , is a phenolic monobasic acid containing two methoxyl groups. It must be a chlorohydroxy-dimethoxybenzoic acid since, with diazomethane, it gave methyl 3-chloro-2:4:6-trimethoxybenzoate identical with that prepared by Calam & Oxford [1939] from phloroglucinol carboxylic acid. Since the griseofulvin degradation product gives a deep purple colour with  $\text{FeCl}_3$  it is clearly a derivative of salicylic acid. Hence it must be either 5-chloro-2-hydroxy-4:6-dimethoxybenzoic acid or 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid, (VII), and since it gives a negative Millon reaction, indicating the absence of an unsubstituted carbon *ortho* to the phenolic group, it must have the latter orientation. The same degradation product is also obtained under similar conditions of oxidation from griseofulvic acid,  $\text{C}_{16}\text{H}_{15}\text{O}_6\text{Cl}$ , and from decarboxygriseofulvic acid,  $\text{C}_{15}\text{H}_{15}\text{O}_4\text{Cl}$ . These facts lead to the following conclusions: (a) griseofulvin itself contains a benzene ring to which are attached two of the three  $\text{—O.CH}_3$  groups and also the chlorine atom; (b) the  $\text{—COO.CH}_3$  group present in griseofulvin is almost certainly not attached to this benzene ring; (c) the  $\text{—COOH}$  and  $\text{—OH}$  groups in the degradation product arise respectively from the oxidation of a  $\text{—C—}$  and a  $\text{—O—}$  linkage. There is present, therefore, in griseofulvin the nucleus



So far as we are aware griseofulvin affords the first recorded instance of the occurrence of the phloroglucinol nucleus in a mould metabolic product.

That griseofulvin, in addition to the above nucleus, also contains a second ring or a long side chain attached to the benzene ring through the  $\text{—C—}$  and  $\text{—O—}$  groups, and containing one or more centres of asymmetry, is indicated by

the second degradation product referred to above. This substance,  $C_{14}H_{15}O_7Cl$ ,  $[\alpha]_{5790} - 24^\circ$ , is a monobasic acid giving no colour with  $FeCl_3$ . It contains two methoxyl groups, a  $—CO—$  group which cannot be present as  $—OC.CH_3$  since the substance does not give iodoform with alkaline iodine and a hydroxyl group which must be tertiary since no reaction is given with the Fearon-Mitchell [1932] reagent for primary and secondary alcohols. On treatment with acetic anhydride and pyridine it did not give an acetate, but the elements of water were eliminated to give a neutral substance,  $C_{14}H_{13}O_6Cl$ . The original substance,  $C_{14}H_{15}O_7Cl$ , is therefore probably a  $\gamma$ -hydroxy-acid of the form

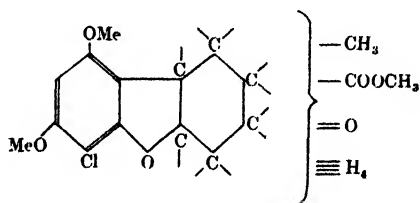


where neither  $R_1$  nor  $R_2$  is hydrogen.

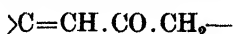
Griseofulvin, on fusion with  $KOH$ , gives orcinol (3:5-dihydroxytoluene, IX). It is difficult, if not impossible, to explain how the substituted benzene ring which yields 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid on oxidation of griseofulvin with  $KMnO_4$  at room temperature could also yield orcinol on  $KOH$  fusion. Hence the conclusion seems irresistible that 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid and orcinol arise from separate halves of the griseofulvin molecule and, as was indicated earlier, that the  $—COOCH_3$  group is attached to the orcinol-yielding half.

Finally, both griseofulvic acid,  $C_{16}H_{15}O_6Cl$ , and *norgriseofulvic* acid,  $C_{15}H_{13}O_6Cl$ , with diazomethane, give not only griseofulvin, identical in its properties with the natural product, but also an *isogriseofulvin*, having the same empirical formula as griseofulvin but with a different m.p. and having  $[\alpha]_{5461} + 265^\circ$  instead of  $+417^\circ$ .

The experimental facts at present available do not lead with certainty to a structural formula for griseofulvin. If, however, it is accepted that 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid and orcinol arise from separate halves of the griseofulvin molecule, and if it is further assumed that orcinol arises from a preformed six-carbon ring with a methyl side-chain attached, then the skeleton



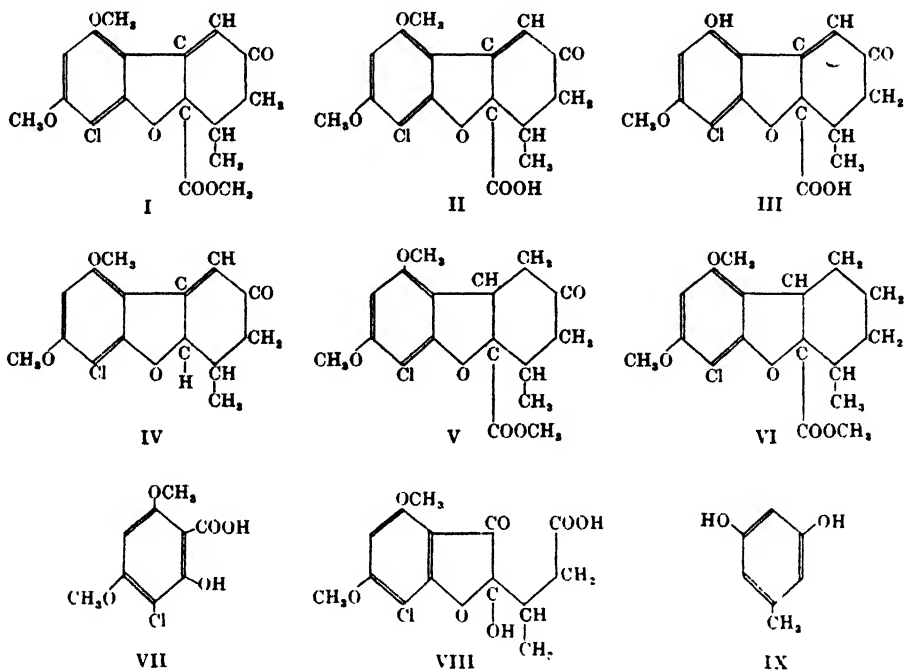
seems inevitable. The non-chlorinated ring must contain the grouping



(see under dihydrogriseofulvin) and also the centre or centres of optical activity. A number of possible structures, which would satisfy the experimental facts available, could be advanced, but of these (I) appears to offer the best working hypothesis and is suggested as a tentative formula.

Accepting (I) for griseofulvin,  $C_{17}H_{17}O_6Cl$ , then griseofulvic acid,  $C_{16}H_{15}O_6Cl$ , would be (II), *norgriseofulvic* acid,  $C_{15}H_{13}O_6Cl$  (III), decarboxygriseofulvic acid,  $C_{15}H_{15}O_4Cl$  (IV), dihydrogriseofulvin,  $C_{17}H_{19}O_6Cl$  (V), tetrahydrodeoxygriseofulvin,  $C_{17}H_{21}O_5Cl$  (VI), and the  $KMnO_4$  oxidation product of griseofulvin,

$C_{14}H_{15}O_7Cl$  (VIII). The known properties of these substances can be readily explained on the basis of the structures proposed for them.



## EXPERIMENTAL

### History of the culture used

The culture of *Penicillium griseo-fulvum* Diereckx used throughout this work was received in December 1929 from Prof. Ph. Biourge of the University of Louvain, and was numbered B. 34. It bears the L.S.H.T.M. Catalogue No. P. 38.

### Cultural conditions

Batches of 100 flasks of the following solution (glucose, 80 g.;  $NaNO_3$ , 2.5 g.;  $KH_2PO_4$ , 1.0 g.;  $KCl$ , 0.5 g.;  $MgSO_4 \cdot 7H_2O$ , 0.5 g.;  $FeSO_4 \cdot 7H_2O$ , 0.02 g.; distilled water, 1000 ml.), 350 ml. in each litre conical flask, were sterilized, sown with a spore suspension of *P. griseo-fulvum*, and incubated at  $30^\circ$  for 65–85 days when the glucose had been reduced to 0.6–0.8%. At the end of the incubation period the mycelium was separated by filtration, washed with cold water, and dried in a vacuum oven at  $50^\circ$ . Weight of dry mycelium from 740 flasks = 2970 g.

### Extraction of griseofulvin

The mycelium was finely ground in a coffee mill, mixed with pumice stone which had been previously washed with ether, and extracted in a Soxhlet apparatus for 3 days with light petroleum (B.P.  $40$ – $50^\circ$ ). The extract consisted of an oil containing small amounts of griseofulvin. The mycelium was then air-dried and re-extracted with ether for 4 days and gave 286 g. of crude solid material = 9.6% of the dried mycelium. This crude solid material consists of a mixture of griseofulvin and a hitherto undescribed mould metabolic product containing nitrogen, which will form the subject of a future communication.



The constituents were separated as follows: 10–15 g. lots were extracted with boiling benzene (350 ml.). The benzene solution, on cooling, deposited the nitrogenous compound in a fairly pure condition. The benzene mother liquors, by evaporation in stages, yielded successive crops of griseofulvin which was purified by crystallization from ethanol. Total yield of pure griseofulvin, 48.9 g.

When *P. griseo-fulvum* was grown on the medium described above but with an equivalent amount of KBr in place of KCl, no metabolic product containing bromine could be isolated although good growth of the mould and metabolism of the glucose occurred. No griseofulvin could be detected in the mycelium of *P. griseo-fulvum* grown on the medium used by Oxford *et al.* [1935] for the isolation of fulvic acid. This medium contains glucose and ammonium tartrate as sources of C and N and KCl as source of Cl.

#### *Analysis and general properties of griseofulvin*

Griseofulvin forms massive, colourless, rhombic crystals by slow deposition from ethanol, m.p. 218–219° without decomposition. (Found: C, 58.05, 57.86; H, 4.93, 4.90; Cl, 9.98, 10.09; CH<sub>3</sub>O, 26.29, 26.91%; N, nil; mol. wt. (cryoscopic in dioxane), 309; (cryoscopic in camphor), 348, 361. C<sub>14</sub>H<sub>8</sub>O<sub>3</sub>Cl(CH<sub>3</sub>O)<sub>3</sub>, i.e. C<sub>17</sub>H<sub>17</sub>O<sub>6</sub>Cl requires C, 57.86; H, 4.85; Cl, 10.06; 3CH<sub>3</sub>O, 26.40%; mol. wt. 352.5.) The substance is quite neutral; 0.0960 g. dissolved in aqueous ethanol required only 0.05 ml. N/10 NaOH for neutralization to phenolphthalein. Optical rotation,  $[\alpha]_{D}^{19} + 354^{\circ}$ ;  $[\alpha]_{D}^{19} + 417^{\circ}$ . ( $c = 1.0127$  in acetone.) It is sparingly soluble in the cold, in chloroform, ethyl acetate, benzene, toluene, ethanol, acetone and dioxane, and is quite insoluble in water. The small solubility in cold dioxane renders an exact determination of the mol. wt. impossible. It gives no colour with ferric chloride in alcoholic solution, but gives a yellow colour without fluorescence in cold conc. H<sub>2</sub>SO<sub>4</sub> and a similar colour in cold conc. HNO<sub>3</sub>. It does not decolor bromine in chloroform solution and gives no reactions with concentrated HBr in glacial acetic acid (not a  $\gamma$ -pyrone) or with alkaline sodium nitroprusside and pyridine (not a  $\beta\gamma$ -unsaturated lactone [Jacobs & Hoffman, 1926]). It cannot be acetylated. It reacts very readily with phenylhydrazine and with 2,4-dinitrophenylhydrazine but crystalline derivatives could not be isolated in a pure state. It does not react with diazomethane or semicarbazide.

*Identification of the alkoxy groups in griseofulvin.* The alkyl iodide from a Zeisel decomposition of griseofulvin (0.1655 g.) was passed into dimethylaniline. The white crystalline precipitate formed (0.3590 g., theoretical for 3CH<sub>3</sub>O groups in griseofulvin 0.3705 g.) behaved on heating in an exactly similar way to authentic dimethylaniline methiodide, i.e. it melted at 206° in a sealed tube and was completely volatilized at 220°. (Found: I, 48.41%. Calc. for C<sub>6</sub>H<sub>5</sub>(CH<sub>3</sub>)<sub>3</sub>NI; I, 48.27%.) All three alkoxy groups in griseofulvin are thus methoxyl groups.

*Griseofulvin mono-oxime.* Griseofulvin (0.7 g.) in ethanol (20 ml.) was refluxed for 7 hr. with hydroxylamine hydrochloride (1.0 g.) and N NaOH (14.3 ml.). On dilution with water the crude oxime separated and was purified by repeated crystallization from benzene-light petroleum. Clusters of shining, colourless leaflets which retained solvent very tenaciously. The air-dried crystals sintered at 120° and melted with gas evolution at 120–40° (probably loss of solvent); the melt reset on further heating and remelted sharply at 226–7° without decomp. (Found, on material dried to constant weight at 110°: C, 55.33; H, 5.23; N, 3.46; Cl, 9.06 CH<sub>3</sub>O, 25.25%. C<sub>17</sub>H<sub>16</sub>O<sub>6</sub>NCl requires

C, 55.49; H, 4.92; N, 3.81; Cl, 9.64;  $3(\text{CH}_3\text{O})$ , 25.31 %.) In spite of the large excess of hydroxylamine used in this preparation there was no evidence of the formation of a di-oxime.

*Catalytic reduction of griseofulvin. Dihydrogriseofulvin and tetrahydrodeoxygriseofulvin*

To a solution of griseofulvin (0.9 g.) in ethyl acetate (140 ml.) were added norite (2.5 g.) and a suspension of powdered  $\text{PdCl}_2$  (0.7 g.) in water (50 ml.) previously heated to boiling and then cooled. The mixture was vigorously shaken with hydrogen and after the initial very rapid uptake of hydrogen required for the reduction of the  $\text{PdCl}_2$  and saturation of the catalyst a slow absorption set in. The reduction was stopped after 7 hr. when 61 ml.  $\text{H}_2$  had been absorbed (theoretical for 1 mol.  $\text{H}_2 = 57$  ml. at N.T.P.). The mixture was filtered, the ethyl acetate layer separated, dried and concentrated to low bulk. Addition of much light petroleum now gave a colourless precipitate (0.7 g., m.p.  $184\text{--}186^\circ$ ) from which were obtained, by fractional crystallization from light petroleum (b.p.  $80\text{--}100^\circ$ ) and aqueous ethanol, two pure substances, dihydrogriseofulvin and tetrahydrodeoxygriseofulvin.

*Dihydrogriseofulvin.* Colourless flat rods, m.p.  $194\text{--}196^\circ$  from light petroleum (b.p.  $80\text{--}100^\circ$ ). (Found: C, 57.60, 57.57; H, 5.31, 5.25; Cl, 10.2 %.  $\text{C}_{17}\text{H}_{19}\text{O}_6\text{Cl}$  requires C, 57.53; H, 5.40; Cl, 10.0 %.) Optical rotation,  $[\alpha]_{540}^{18} - 33^\circ$ ;  $[\alpha]_{579}^{18} - 27^\circ$ . ( $c = 0.452$  in acetone.) Dihydrogriseofulvin is more readily soluble in most organic solvents than griseofulvin. It gives only a yellow colour with  $\text{FeCl}_3$  in alcoholic solution. Unlike griseofulvin, which gives no colour, it gives a deep rose red colour on warming with salicylaldehyde and 45 % aqueous  $\text{H}_2\text{SO}_4$ . Addition of Brady's reagent [1931] to an alcoholic solution gives a yellow crystalline precipitate, m.p.  $264\text{--}266^\circ$ , which is probably a mono-2:4-dinitrophenylhydrazone since it gives a reddish brown colour with alcohol and a trace of aqueous KOH.

*Tetrahydrodeoxygriseofulvin.* Long colourless slender needles, m.p.  $180^\circ$ , from aqueous ethanol or methanol. (Found: C, 59.99, 60.00; H, 6.28, 6.43; Cl, 10.5, 10.6;  $\text{CH}_3\text{O}$ , 26.9 %.  $\text{C}_{17}\text{H}_{21}\text{O}_5\text{Cl}$  requires C, 59.88; H, 6.22; Cl, 10.4;  $3\text{CH}_3\text{O}$ , 27.3 %.) The substance is quite insoluble in dilute KOH. It gives an intense greenish yellow colour with cold concentrated  $\text{H}_2\text{SO}_4$  becoming wine-red on warming. A solution in aqueous alcohol gives no precipitate with Brady's reagent even on heating. It is not hydrolysed by boiling for 4 hr. with aqueous-alcoholic  $\text{N H}_2\text{SO}_4$  or by boiling for 7 hr. with  $\text{N}/2$  NaOH, facts which are difficult to explain unless a steric hindrance effect is assumed.

*Hydrolysis of griseofulvin*

(A) *With dilute aqueous-alcoholic  $\text{H}_2\text{SO}_4$ . Griseofulvic acid.* A mixture of griseofulvin (1.0 g.), ethanol (200 ml.) and  $2\text{N H}_2\text{SO}_4$  (250 ml.) was refluxed for 6 hr. The ethanol was removed *in vacuo*, the colourless precipitate collected, washed with water and then with dilute  $\text{Na}_2\text{CO}_3$  in which it was almost completely soluble. The alkaline solution was acidified and the resulting precipitate (0.8 g.) was crystallized from boiling aqueous methanol in which it is only sparingly soluble. Colourless, flat, hexagonal prisms, m.p.  $256\text{--}260^\circ$ . (Found: C, 56.85, 56.77; H, 4.54, 4.61; Cl, 10.38, 10.14;  $\text{CH}_3\text{O}$ , 18.2 %. Equiv. by titration, 362.  $\text{C}_{16}\text{H}_{15}\text{O}_6\text{Cl}$  requires C, 56.70; H, 4.46; Cl, 10.47;  $2\text{CH}_3\text{O}$ , 18.3 %. Equiv., as a monobasic acid, 339.) Optical rotation,  $[\alpha]_{540}^{18} + 508^\circ$ ;  $[\alpha]_{579}^{18} + 420^\circ$ . ( $c = 0.2256$  as the sodium salt in aqueous methanol.)

Griseofulvic acid gives a yellowish brown colour with  $\text{FeCl}_3$ , a yellow colour with cold conc.  $\text{H}_2\text{SO}_4$ , and only a faint yellow colour with diazotized sulphanilic acid in  $\text{Na}_2\text{CO}_3$  solution. The Millon reaction is also negative. It is readily soluble in aqueous  $\text{NaHCO}_3$  and the resulting solution slowly decolorizes very dilute  $\text{KMnO}_4$  solution. It gives a dark red amorphous precipitate with Brady's reagent.

(B) *With dilute aqueous-alcoholic NaOH.* A mixture of griseofulvin (2.25 g.), ethanol (300 ml.) and  $N/4$   $\text{NaOH}$  (200 ml.) was refluxed for 3 hr. The solution was cooled, acidified, the ethanol removed *in vacuo* and the colourless precipitate collected, washed and dried (2.3 g.). It was crystallized from aqueous methanol in colourless hexagonal prisms, M.P.  $255-262^\circ$ , alone or in admixture with griseofulvic acid formed on acid hydrolysis of griseofulvin (see (A) above). Equiv. by titration, 374.  $\text{C}_{16}\text{H}_{15}\text{O}_6\text{Cl}$ , as a monobasic acid, requires 339.

(C) *With dilute aqueous NaOH.* A mixture of griseofulvin (3.82 g.),  $N$   $\text{NaOH}$  (250 ml.) and water (350 ml.) was refluxed for 5 hr. At first part of the solid appeared to dissolve, but later much colourless material separated. Next day this was collected, washed and dried (fraction I, wt. 1.04 g.). The alkaline filtrate was acidified, filtered from a little dark coloured amorphous material and set aside. An almost colourless, crystalline substance (fraction II, wt. 1.6 g.) separated.

*Purification and properties of fraction I. Decarboxygriseofulvic acid.* Colourless needles from aqueous ethanol, M.P.  $138-140^\circ$ . Contains chlorine. (Found: C, 61.25, 61.15; H, 5.09, 5.10;  $\text{CH}_3\text{O}$ , 21.01, 21.37; mol. wt. (cryoscopic in dioxane), 279. Equiv. nil.  $\text{C}_{15}\text{H}_{15}\text{O}_4\text{Cl}$  requires C, 61.10; H, 5.13;  $2\text{CH}_3\text{O}$ , 21.05%; mol. wt. 295.) Optical rotation,  $[\alpha]_{5461}^{18} - 31^\circ$ . ( $c = 0.163$  in acetone.)

Decarboxygriseofulvic acid is much more soluble in the usual organic solvents than is griseofulvin. It gives no colour with  $\text{FeCl}_3$  in aqueous alcoholic solution. With cold conc.  $\text{H}_2\text{SO}_4$  it gives an immediate intense yellow colour, quickly changing to brown and finally to a deep purple (permanganate) colour. It gives a brownish red colour with saturated alcoholic picric acid but no picrate separates. It is unchanged by boiling with dilute aqueous-alcoholic  $\text{H}_2\text{SO}_4$ .

*Purification and properties of fraction II. norGriseofulvic acid.* Long, colourless, pointed needles from aqueous methanol, M.P.  $260^\circ$  decomp. (Found: C, 55.67; H, 4.18; Cl, 10.46, 10.61;  $\text{CH}_3\text{O}$ , 10.6%. Equiv. by titration, 165.  $\text{C}_{15}\text{H}_{13}\text{O}_6\text{Cl}$  requires C, 55.46; H, 4.04; Cl, 10.92;  $1\text{CH}_3\text{O}$ , 9.6%. Equiv. as a dibasic acid, 162.) Optical rotation,  $[\alpha]_{5461}^{18} + 609^\circ$ ;  $[\alpha]_{5790}^{18} + 505^\circ$ . ( $c = 0.2262$  as sodium salt in water.) *norGriseofulvic acid* is readily soluble in hot methanol but is almost insoluble in boiling chloroform. It gives a positive Millon reaction, an intense brown colour with  $\text{FeCl}_3$  in aqueous methanol, an intense orange brown colour with diazotized sulphanilic acid in  $\text{Na}_2\text{CO}_3$  and a yellow colour with cold conc.  $\text{H}_2\text{SO}_4$ . It was recovered unchanged after boiling with  $N$  aqueous-alcoholic  $\text{H}_2\text{SO}_4$ .

(D) *Hydrolysis of griseofulvic acid with  $N/2$  aqueous NaOH.* Griseofulvic acid ( $\text{C}_{16}\text{H}_{15}\text{O}_6\text{Cl}$ , ((A) above) 0.53 g.) was boiled with  $N/2$  aqueous  $\text{NaOH}$  (70 ml.) for 4 hr. The products of hydrolysis were decarboxygriseofulvic acid,  $\text{C}_{15}\text{H}_{15}\text{O}_4\text{Cl}$  ((C) above, fraction I, 0.13 g., M.P.  $138-140^\circ$  alone or in admixture) and *norgriseofulvic acid*,  $\text{C}_{15}\text{H}_{13}\text{O}_6\text{Cl}$  ((C) above, fraction II, 0.15 g., M.P.  $260^\circ$  alone or in admixture).

*Methylation of griseofulvic acid and norgriseofulvic acid with diazomethane*

*norGriseofulvic acid*,  $\text{C}_{15}\text{H}_{13}\text{O}_6\text{Cl}$  (0.38 g.), suspended in ether was methylated by the addition of diazomethane prepared from nitrosomethylurethane (7 ml.). After several hours the solution was separated from a little insoluble precipitate,

the solvent removed and the crystalline residue (0.43 g.) fractionally crystallized from ethanol. The more soluble component proved to be griseofulvin. Prisms, m.p. 217–218°, alone or in admixture with authentic griseofulvin. The less soluble component proved to be an *isogriseofulvin*, long colourless needles from ethanol (0.15 g.), m.p. 198–200°, and giving a large depression on admixture with griseofulvin. (Found: C, 57.82, 57.88; H, 4.98, 4.99; N, nil; Cl, 10.20; CH<sub>3</sub>O, 26.51%. C<sub>17</sub>H<sub>17</sub>O<sub>6</sub>Cl requires C, 57.86; H, 4.85, Cl, 10.06; 3CH<sub>3</sub>O, 26.40%.) Optical rotation,  $[\alpha]_{5461}^{100} + 265^\circ$ ;  $[\alpha]_{5790}^{100} + 223^\circ$ . ( $c = 0.1424$  in acetone.) It is very similar in all its properties to griseofulvin except that it is less soluble in organic solvents.

Methylation of griseofulvic acid, C<sub>16</sub>H<sub>15</sub>O<sub>6</sub>Cl, with diazomethane also gave a mixture of griseofulvin and *isogriseofulvin*, though in this case griseofulvin was formed in the larger amount.

#### *Oxidation of griseofulvin by KMnO<sub>4</sub> in acetone*

Griseofulvin (4 g.) dissolved in pure acetone (1 litre) was treated with finely powdered KMnO<sub>4</sub> (16 g.). The mixture was kept cool and shaken occasionally during 5 hr. The MnO<sub>2</sub> and insoluble potassium salts formed were separated, washed with acetone, dried and ground with dilute ammonia. The filtered ammoniacal solution was acidified to Congo red with dilute H<sub>2</sub>SO<sub>4</sub>. A gum was precipitated which later solidified (0.8 g.). (For treatment of filtrate from gum, see below.) The gum was repeatedly crystallized from ethyl acetate to yield 0.2 g. of colourless needles, m.p. 224° decomp., of 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid. (Found: C, 46.52, 46.49; H, 4.01, 3.96; Cl, 15.36, 15.64; CH<sub>3</sub>O, 26.18, 26.42%. Equiv. 231. C<sub>9</sub>H<sub>9</sub>O<sub>5</sub>Cl requires C, 46.45; H, 3.90; Cl, 15.25; 2CH<sub>3</sub>O, 26.67%. Equiv. as a monobasic acid, 232.5.) This acid is insoluble in water and sparingly soluble in all organic solvents. It gives a beautiful purple colour with FeCl<sub>3</sub> in alcoholic solution but gives a negative Millon reaction.

0.3 g. of the acid, on methylation with ethereal diazomethane, yielded 0.15 g. of the methyl ester of 3-chloro-2:4:6-trimethoxybenzoic acid [Calam & Oxford, 1939]. Colourless hexagonal platelets from light petroleum, m.p. 127–128°, alone or in admixture with an authentic specimen. (Found: C, 50.55, 50.40; H, 5.01, 5.04; CH<sub>3</sub>O, 47.20, 46.79%. Mol. wt., cryoscopic in dioxane, 248. C<sub>11</sub>H<sub>13</sub>O<sub>5</sub>Cl requires C, 50.67; H, 5.03; 4CH<sub>3</sub>O, 47.63%; mol. wt. 260.5.) The substance is quite insoluble in KOH solution and gives no colour with FeCl<sub>3</sub>.

3-Chloro-2-hydroxy-4:6-dimethoxybenzoic acid is also formed by the oxidation with KMnO<sub>4</sub> in acetone of griseofulvic acid, C<sub>16</sub>H<sub>15</sub>O<sub>6</sub>Cl, and decarboxy-griseofulvic acid, C<sub>15</sub>H<sub>15</sub>O<sub>4</sub>Cl.

The filtrate from the gum referred to above was extracted with ether. On removal of the solvent a small amount of colourless material remained which, on crystallization from benzene-light petroleum-chloroform, yielded clusters of minute rods, m.p. 200° decomp. (Found: C, 50.70, 50.79; H, 4.48, 4.45; Cl, 10.7, 11.1; CH<sub>3</sub>O, 18.4, 18.3%. Equiv., 320. C<sub>14</sub>H<sub>15</sub>O<sub>7</sub>Cl requires C, 50.82; H, 4.57; Cl, 10.73; 2CH<sub>3</sub>O, 18.77%. Equiv. as a monobasic acid, 330.5.) Optical rotation,  $[\alpha]_{5790}^{100} - 24^\circ$ . ( $c = 0.8506$  as the sodium salt in 20% aqueous methanol.) The acid is appreciably soluble in water, readily so in ethanol, and gives no colour with FeCl<sub>3</sub>. It appears to contain a —CO— group since, although its aqueous solution does not give an immediate reaction with Brady's reagent, a good yellow precipitate forms after 2 days. It also appears to contain an OH group since, on treatment with acetic anhydride in pyridine at 37° for several days, although no acetyl derivative is formed, the elements of water are eliminated to give a *neutral substance*, C<sub>14</sub>H<sub>13</sub>O<sub>6</sub>Cl, as colourless needles from aqueous ethanol,

M.P. 220° (no decomp.). (Found: C, 53.87; H, 4.18; Cl, 11.14%.  $C_{14}H_{13}O_6Cl$  requires C, 53.74; H, 4.19; Cl, 11.34%.) The acid  $C_{14}H_{15}O_7Cl$  gives a negative iodoform reaction with alkaline iodine, a negative Fearon-Mitchell reaction [1932] for primary and secondary alcohols and it does not appear to contain a lactone grouping since treatment with excess NaOH for some time at 37° does not unmask additional acidity. It reduces Fehling's solution on boiling. It is also formed by the  $KMnO_4$  oxidation of griseofulvic acid but not of decarboxy-griseofulvic acid.

#### *Fusion of griseofulvin with KOH*

Griseofulvin (1 g.) was fused for 1 hr. at 225–50° with solid KOH (3.5 g.) and water (1.5 ml.) in a nickel crucible. The melt was cooled and dissolved in water, and the solution saturated with  $CO_2$  and extracted with ether. Evaporation of the dried extract gave a colourless crystalline residue (0.26 g.), which, when fractionally crystallized from light petroleum gave three fractions melting at 55, 95 and 107°. All three fractions gave the characteristic reactions of orcinol, i.e. a neutral aqueous solution giving a bluish purple colour with  $FeCl_3$ , a red colour with sodium hypochlorite and with ammonia and a precipitate with bromine water. A pink colour with a strong green fluorescence was observed when any of the fractions was dissolved in caustic potash and shaken with chloroform. The first fraction was hydrated orcinol (M.P. 58°) and the other two essentially the anhydrous phenol (M.P. 107°). All three fractions, on sublimation in a high vacuum at 55–60° gave a sublimate melting at 96°, not depressed on admixture with authentic anhydrous orcinol. (Found: C, 67.39, 67.38; H, 6.69, 6.40%. Mol. wt. (in camphor), 129, 130.  $C_7H_6O_2$  requires C, 67.70; H, 6.49; mol. wt. 124.)

#### SUMMARY

Griseofulvin,  $C_{17}H_{17}O_6Cl$ , a hitherto undescribed mould metabolic product, has been isolated from the mycelium of *Penicillium griseo-fulvum* Dierckx grown on a modified Czapek-Dox solution. The general properties of griseofulvin are described together with a number of derivatives and degradation products. A provisional structural formula for griseofulvin is suggested which illustrates the experimental findings.

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# XXX. STUDIES ON THE METABOLISM OF PYRUVIC ACID IN NORMAL AND VITAMIN B<sub>1</sub>-DEFICIENT STATES

## I. A RAPID, SPECIFIC AND SENSITIVE METHOD FOR THE ESTIMATION OF BLOOD PYRUVATE

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SINCE the appearance of the Meyerhof-Embden scheme pyruvic acid is known to be a normal intermediate metabolite of the carbohydrate breakdown of mammalian tissues, of yeast fermentation and of the respiration of higher plants. It is involved in the metabolism of embryos, bacteria and tumour tissues, and in the action of hormones and vitamins. It also forms a link between the metabolism of carbohydrate and amino-acids. For the study of pyruvate metabolism in man with relation to vitamin B<sub>1</sub>-deficiency, however, a really suitable method has not hitherto been available.

No less than fifty descriptions of methods or modifications of previous methods for the identification and quantitative estimation of pyruvic acid have been reviewed by Wendel\* [1932]. Since then numerous other methods have been described; there is the NaHSO<sub>3</sub>-binding method of Clift & Cook [1932], the 2:4-dinitrophenylhydrazone method of Case\* [1932], the carboxylase method for determination in blood [Westerkamp, 1933], the modification of the Neuberg-Case method [Peters & Thompson, 1934], the semicarbazide-HCl precipitation method and its modification [Hahn *et al.* 1934; 1936], the  $\alpha$ -methylindole-HCl colour reaction for its estimation in blood [Dische & Robbins, 1934], the ceric sulphate method [Fromageot & Desnuelle, 1935], the red colour reaction of the 2:4-dinitrophenylhydrazone of pyruvic acid in alkaline solution [Jowett & Quastel, 1937], and finally the microchemical adaptation of the method of Clift & Cook for use with minute quantities of blood by de Jong & Picard\* [1937].

Of all the methods mentioned above only a few are applicable to quantitative blood analysis because of the sensitivity required. Pyruvic acid is present in normal blood in extremely small amounts: below 0.6 mg./100 ml. The carboxylase method, apart from its questionable specificity, is too complicated for ordinary purposes. The ceric sulphate method is simple and rapid for estimating small quantities; but the interference of lactic acid prevents it from being adequate for analysis of biological fluids. Unfortunately neither of the two micro-methods described is really specific. The  $\alpha$ -methylindole-HCl reaction of Dische & Robbins has been found by de Jong & Picard to be non-specific; the intensity of the colour developed varying with the concentration of the reagents and temperature. The author agrees with Peters & Thompson [1934] in finding that heating with alkali to remove substances other than pyruvic acid as described by Clift & Cook is not satisfactory when tissue or blood extracts are used. Many experiments of this type were done on beri-beri blood and the value never

\* Criticisms of some of the previous methods are to be found in references marked with an asterisk.



agreed with that obtained by the elaborate hydrazone method: hence the micromethod described by de Jong & Picard may, like the estimation of bisulphite-binding substances, work well with pigeons' blood, but is not applicable to clinical studies. This leaves only the hydrazone method. It is specific, but suffers from the following disadvantages. First, for clinical purposes the procedure is complicated; secondly, the length of time required often renders it of no value to the physician, either for diagnosis or treatment, since acute beriberi patients usually die a few hours after admission; and thirdly the venous puncture necessary in order to obtain enough blood (at least 1.2 ml.) for every test may be an important obstacle to research studies on the changes in human or animal blood pyruvate. It is therefore necessary to have a method which is simple, specific, rapid and needs only small quantities of blood.

The difficulty in the quantitative estimation of pyruvic acid is the impossibility of separating it from other aldehyde or ketone derivatives present. In colorimetric reactions these compounds form colours which are liable to modify the tint. It is possible to develop a colorimetric determination of pyruvic acid in small quantities by the selection of optimal specific conditions in which the colour of the pyruvic acid hydrazone is maximal and permanent, and by the use of a light filter.

The principle is as follows: pyruvic acid is first converted into its 2:4-dinitrophenylhydrazone. This is extracted with ethyl acetate from the aqueous solution. The hydrazone of pyruvic acid is separated from the excess of hydrazine added and the other hydrazones of aldehyde or ketone derivatives by extraction with  $\text{Na}_2\text{CO}_3$ . Traces of the hydrazine or hydrazone carried over by the ethyl acetate dissolved in the aqueous  $\text{Na}_2\text{CO}_3$  are removed with a fresh lot of ethyl acetate. The stable red colour developed by adding  $\text{NaOH}$  to the  $\text{Na}_2\text{CO}_3$  extract of the 2:4-dinitrophenylhydrazone of pyruvic acid is determined colorimetrically, using a photoelectric colorimeter.<sup>1</sup> The interference of the yellow colour is eliminated by the use of a light filter.

Reagents required:                      *Experimental details*

5 % and 10 % trichloroacetic acid,  
0.1 % 2:4-dinitrophenylhydrazine in 2 N HCl,  
Ethyl acetate, pure,  
N NaOH,  
10 %  $\text{Na}_2\text{CO}_3$ .

2 or 3 drops of freshly shed blood are dropped directly into an accurately weighed 10 ml. centrifuge tube containing 1.0 ml. 10 % trichloroacetic acid and previously cooled to below 0°. After mixing well, it is weighed again to  $\pm 0.1$  mg. or it may be convenient to use the 0.2 ml. special blood pipette described by Harrison [1937] for micro-sugar analysis. The precipitated protein is centrifuged down 10 min. after the blood is taken. The clear supernatant liquid is transferred quantitatively to an ordinary test tube (tube 1). The protein precipitate is mixed with 1 ml. 5 % trichloroacetic acid, and after being allowed to stand for 2 min. is centrifuged again and the supernatant liquid is transferred to tube 1. To the combined trichloroacetic extracts is added 1 ml. 2:4-dinitrophenylhydrazine solution. The mixture is allowed to stand at room temp. for not less than 10 min. It is then extracted with 2 ml. ethyl acetate and well mixed. When the two layers have separated out, the lower acid layer is quantitatively

<sup>1</sup> This is made by the Unicam Instrument Company, Cambridge, on the lines of the Evelyn Colorimeter [Evelyn, 1936].

removed with a fine-tipped dropper to tube 2, and is there extracted again with 1.0 ml. ethyl acetate. The upper ethyl acetate layer is carefully transferred to tube 1, and the lower layer is extracted for a third time with 1.0 ml. ethyl acetate. The clear aqueous layer is now discarded and the ethyl acetate added to tube 1. The combined ethyl acetate extract contains all the unchanged hydrazine added, and the hydrazones formed. From a burette exactly 2.0 ml.  $\text{Na}_2\text{CO}_3$  are added to tube 2, rinsed well and the whole poured into tube 1. This is then mixed well with the aid of the same dropper and allowed to extract for at least 3 min. When the separation is completed the  $\text{Na}_2\text{CO}_3$  layer is returned to tube 2. This extraction with  $\text{Na}_2\text{CO}_3$  is repeated twice again with 2 ml. portions of  $\text{Na}_2\text{CO}_3$  each time. The combined  $\text{Na}_2\text{CO}_3$  solution in tube 2 is extracted for the last time with 1.0 ml. of ethyl acetate. The clear  $\text{Na}_2\text{CO}_3$  extract, which may or may not be coloured yellow, depending on the quantity of the hydrazone present, is quantitatively transferred to a clean tube 3 to which 4.0 ml.  $N$  NaOH are added. A stable red colour develops which is determined after 10 min., using a photoelectric colorimeter with Wratten No. 62 light filter. The exact amount of pyruvic acid present in the given sample is read off from the standard curve.<sup>1</sup>

The standard curve is constructed as follows. A pure preparation of the 2:4-dinitrophenylhydrazone dissolved in ethyl acetate solution can be used, but a pure pyruvate solution is preferred. The pure pyruvic acid solution is prepared by three redistillations *in vacuo*, the fraction boiling at 55–60°/10 mm. Hg being collected. The clear liquid crystallizes out on standing in a freezing mixture at  $-4^\circ$ . The crystals, carefully weighed in a weighing bottle, are dissolved in 50 vol. of ice-cold freshly distilled water. After neutralization with NaOH,

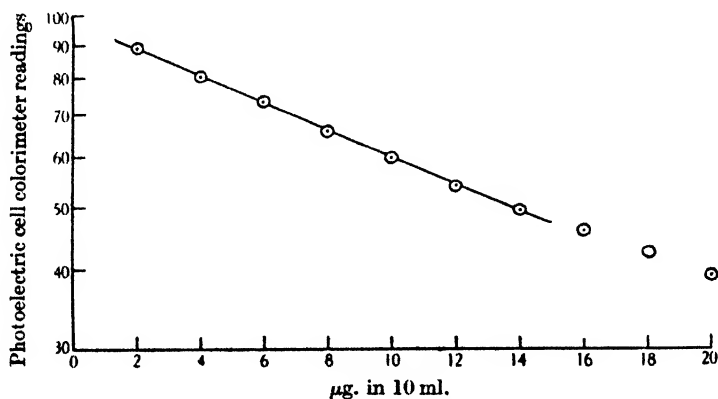


Fig. 1. Standard curve.

care being taken not to let the temperature rise, the pyruvate solution is diluted to 1%. The exact concentration of the solution is checked by estimating its bisulphite-binding power. The standard pyruvate solution thus prepared is further diluted to a concentration of 5  $\mu\text{g.}/\text{ml.}$  Duplicate series of tubes, containing pyruvic acid from 2 to 20  $\mu\text{g.}$ , are set up, and estimations carried out in the manner described above for blood analysis. The red colour developed is stable from the first min. for more than 90 min. in solutions of pure pyruvate

<sup>1</sup> It is always necessary to prepare a blank of trichloroacetic acid and hydrazine, extracted in the usual manner, to be used as a control solution for setting the zero of the photoelectric colorimeter. If the experiment is well carried out the blank is practically colourless every time. After each reading the galvanometer should return to the original mark.





hydrazone. The deflexions of the galvanometer readings when plotted against concentrations of pyruvic acid on arithlog paper give a straight line (Fig. 1).

The procedure just described requires a few further comments. As the method is extremely sensitive, it is essential to take every precaution to prevent loss of traces of the extract containing the pyruvic acid 2:4-dinitrophenylhydrazone by avoiding the use of a separating funnel or of numerous test tubes. We differ from previous authors, who worked on a much larger scale and in presence of a great excess of 2:4-dinitrophenylhydrazine, in finding that the  $\text{Na}_2\text{CO}_3$  under the conditions described completely removes the pyruvic acid derivative as shown by the recovery data. Moreover, mixing gently with the aid of the dropper instead of shaking in a separating funnel gives rise to no foaming and hence no interference with the perfect separation of the two layers.  $\text{Na}_2\text{CO}_3$  is to be preferred to  $\text{Na}_2\text{HPO}_4$  as the latter gives a much inferior colour at the last stage. The combined techniques of extracting with ethyl acetate to remove impurities at an alkaline reaction, using a comparatively small but sufficient amount of hydrazine and developing the red colour in the aqueous  $\text{Na}_2\text{CO}_3$  extract of the hydrazone enable us to eliminate the subsequent neutralizations and re-extractions with ethyl acetate, and the drying at the last stage, which are so laborious and liable to introduce error. 2:4-Dinitrophenylhydrazones of other keto-acids also give a red colour, but in the concentrations present in human biological fluids, whether normal or in ketosis, these substances do not interfere. Under the experimental conditions described, if they are present in larger amounts, their colour fades into yellow on standing at room temp. for 10 min. after the addition of  $\text{NaOH}$ . This reaction as used by Jowett & Quastel is not applicable to the estimation of pyruvic acid changes for studies on the physiology or pathology of animals, because the changes in blood pyruvic acid are so small [Thompson & Johnson, 1935; Platt & Lu, 1935; 1936] and the method is not specific enough.

A comparison of pyruvate values was made between the present method and the modified Neuberg-Case hydrazone method [Peters & Thompson, 1934]. The results are shown in Table I.

Table I

mg. per 100 g.

Specimen used	Macro-method (Neuberg-Case)	New micro-method	Difference	Micro-method % of macro-method
Blood	0.765	0.770	0.005	101
Milk	0.584	0.570	0.014	98
Cerebro-spinal fluid	0.940	0.951	0.011	101

From these figures it is obvious that the methods are in good agreement.

#### *Sensitivity and specificity of the method*

The method estimates 2  $\mu\text{g.}$  in 10 ml., a dilution of 1 : 5,000,000 with an error of  $\pm 1.5\%$ . It is 1000 times more sensitive than the reaction of Simon & Piaux [1924] and 50 times more sensitive than the method of Case [1932]. Since the same principle of isolating the hydrazone of keto-acids by carbonate extraction is used in the new method its specificity is at least as high as that of the Neuberg-Case method. Indeed the use of the light filter has rendered it much more specific when small amounts of other keto-acids have been added. How far this is important for practical purposes in studies of blood in diseased conditions is not yet determined.

For many years acetoacetic acid has been known to increase in the blood in ketosis. Recently Krebs [1938] has found  $\alpha$ -ketoglutaric acid to be present in human blood and urine, while oxaloacetic acid has been found by numerous authors to play a part in tissue metabolism. It is possible that laevulic and glucuronic acids may also prove to be of physiological importance. It may therefore be necessary to determine the total amount of keto-acids present. This may be done by substituting 15 %  $\text{Na}_2\text{CO}_3$  for 10 %, by using 3 *N* instead of *N* NaOH, and by taking the reading 2 min. after mixing. Under these conditions all the keto-acids give maximum colour.

On the other hand, under the conditions described in this paper, where pyruvic acid alone is to be estimated, the relative interference is as follows:

	Deflexion of the photo-cell colorimeter reading equivalent to 1 $\mu\text{g}$ . pyruvic acid
Acetoacetic acid	59 $\mu\text{g}$ .
Laevulic acid	15 "
$\alpha$ -Ketoglutaric acid	7.5 "

Oxaloacetic acid, if present, is of course always estimated as pyruvic acid, being unstable in acid solution. The total keto-acids could be estimated after a pyruvic acid determination by adding concentrated  $\text{Na}_2\text{CO}_3$  and NaOH to make the final proportions as indicated above, and then measuring the colour again in the colorimeter. In this case, however, an appropriate calibration curve would have to be worked out.

The present method possesses yet further advantages over the standard Neuberg-Case method, for whereas the application of the latter to urine often leads to yellowish brown colours and gives purplish ones with tissue extracts, none of these inconveniences are met with in the present method.

Application of the method to the analysis of cerebro-spinal fluid, milk, urine and muscle involves no change except in the preparation of the pyruvic acid extract. Deproteinization of cerebro-spinal fluid and urine is carried out in the same manner as with blood. For tissue 8 parts of 5 %  $\text{CCl}_3\text{COOH}$  are used for every g. In the case of milk, 1 ml. of 75 %  $(\text{NH}_4)_2\text{SO}_4$  is used in place of 1 ml. 10 % and 5 % trichloroacetic acid. Tables II and III illustrate the recoveries of added pyruvate in blood and milk.

Table II. *Recovery of pyruvic acid from human blood*

Pyruvic acid found in 100 ml. blood mg.	Pyruvic acid added per 100 ml. mg.	Pyruvic acid recovery	
		Found mg./100 ml.	Calc. mg./100 ml.
0.56 (normal)	2.0	2.52	2.56
0.98 (subacute beri-beri)	1.0	1.93	1.98
2.35 (acute beri-beri)	0.5	2.82	2.85

Table III. *Recovery of pyruvate from milk*

Pyruvic acid found in 100 ml. milk mg.	Pyruvic acid added per 100 ml. mg.	Pyruvic acid recovery	
		Found mg./100 ml.	Calc. mg./100 ml.
0.24	0.75	0.98	0.99
0.34	1.00	1.34	1.34
0.53	0.50	1.06	1.03

Recoveries from tissue extracts are of the same order.

## SUMMARY

The colour of the 2:4-dinitrophenylhydrazone of pyruvic acid may be used for the rapid, specific and sensitive micro-estimation of this substance in biological fluids if new extraction procedures for removing interfering substances are adopted, and if a light filter is used in the photoelectric cell colorimeter to increase accuracy by eliminating accompanying traces of other tints.

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# XXXI. THE PROTEINS OF ELECTRICAL TISSUE

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As a corollary to the more general study of muscle proteins [Bailey, 1937, 2] an investigation of the proteins of electrical tissue is of especial interest, for the electrical organs of some elasmobranch fish in the genus *Raiu* and *Torpedo* arise from the same embryonic rudiments as those of muscle, and earlier studies by Ewart have shown that the development of the electrical layer takes place at the expense of the fibrils. In the primitive organ of *R. radiata*, for instance, the metamorphosis to electrical tissue is only partially complete, the muscle fibrils developing into club-shaped cells bearing a richly innervated cup-like electrical layer and exhibiting the characteristic striations of skeletal muscle [Ewart, 1888]; in *R. circularis* and *R. fullonica* the electric cups are retained, while in *R. batis* they have developed into discs, and in all these three latter species the striations are no longer observable [Ewart, 1892]. It is in *R. clavata* and *Torpedo*, however, that the change is most marked, for here the electrical organs consist of cones or columns of cells separated by septa identical with those between the myotomes of muscle and each cell carries a nerve plate (i.e. the electrical layer), an alveolar layer and a mucilaginous material of high refractive index.

The metamorphosis of muscle rudiments to richly innervated discs suggests that the organ is essentially a modified muscle in which the contractile mechanism, generally considered to be the protein myosin, has disappeared, and that the reactions supplying electrical energy in the one case and contractile energy in the other are comparable. This supposition is supported by experiments of Baldwin & Needham [1937] and of Baldwin [1938] who find that the cyclical reactions of the glycolytic system, which are intimately associated with the liberation of energy in muscle working anaerobically, are catalysed by enzymes also common to *R. clavata* and *Torpedo*, the two species which they studied. To supplement this hypothesis, an investigation of the protein components and their comparison with those of skeletal muscle have been undertaken, and an attempt has been made to show whether the striations in the organ of *R. radiata* are coincidental with the presence of myosin, whether other globulins are also present and whether these latter have disappeared from the organs of *R. clavata* and *Torpedo* where metamorphosis from muscle rudiments to electrical tissue is most complete. The tissues have thus been compared with muscle with particular reference to their N partition, the physico-chemical properties and cataphoretic isoelectric point of the fractionated proteins, and in the case of *Torpedo* protein chemical analyses are presented.

Previous chemical studies are relatively sparse, and almost solely devoted to species of *Torpedo*. An investigation undertaken by J. Davy [1832] at the request of Sir Humphry Davy records for the tissue the low total solid content of 7%, whilst Matteucci [1838] gives figures of 9.2 and 10.4%; the latter found in the organ lactic acid, fatty substances, protein, traces of "gelatin", sodium



and potassium. Frerichs & Städeler [1858] discovered urea and Schultze [1859] reported creatinine, lactic acid, calcium phosphate, sodium chloride, sulphate and a mucous substance precipitated by acetic acid. The tissue insoluble in water he identified as fibrous tissue. Weyl [1881; 1883; 1887] added to this list nuclein, xanthine, hypoxanthine, cholesterol and made a detailed analysis of the ash. He prepared the mucin from the alcohol- and ether-extracted organ by treatment with dilute NaOH and subsequent precipitation with dilute acetic acid. After purification the product gave the following analysis: C, 52.5; H, 7.2; N, 13.2; S, 1.03%. Baglioni [1906] carried out a proximate analysis of the tissue, recording a glycogen content of 0.09% of the fresh weight. Later investigations have centred upon the phosphagen content [Kisch, 1930; Baldwin, 1933] and on the role of adenylic compounds [Baldwin & Needham, 1937; Baldwin, 1938].

### *Experimental*

*Nitrogen partition of the tissues.* The methods evolved by Smith [1934; 1937] and by Reay & Kuchel [1936] for the partition of N between the proteins of skeletal muscle were adopted with minor variations. The total coagulable N (T.C.N.) was determined by mixing a weighed aliquot of minced tissue suspended in water with an equal volume of 10% trichloroacetic acid. After centrifuging, the protein was washed 4–5 times with 5% trichloroacetic acid until free from the non-coagulable nitrogenous substances, which, occurring mainly as urea, are present in large amounts in elasmobranch tissue. The N of the washed protein was determined by the Kjeldahl method. Another aliquot of tissue was exhaustively extracted at 0° by grinding with silver sand in presence of 7% LiCl; the extract was separated by centrifuging, the grinding repeated at least 10 times, and the combined extracts were adjusted with salt solution to a volume containing 0.02–0.04 g. N/100 ml., and the protein in aliquots of this solution was determined by precipitating with trichloroacetic acid as before. Other portions of the tissue in certain instances were exhaustively extracted with  $N/20$  NaOH and  $N/50$  HCl.

In the case of skeletal muscle, the proteins dissolving in the LiCl solution represent the major portion of the intracellular globulins and albumins, viz. myosin, globulin X and myogen, but the residual intracellular fraction which, according to Smith [1937], consists mainly of denatured globulin X, dissolves, together with the salt-soluble intracellular fraction, in dilute acid or alkali; the protein undissolved by these latter is the muscle stroma. Myosin is estimated by dilution of the LiCl extract with 20 vol. of ice-cold water and the gelatinous precipitate which settles out overnight at 0.07–0.08 *M* LiCl is centrifuged off, washed with water and digested in the Kjeldahl flask. The non-coagulable N has been estimated in the LiCl and other extracts by subtracting the protein-N from the total N of the extract.

In Table I a detailed comparison between the N partition of the skeletal muscle and that of electrical tissue is made for three separate specimens of *Torpedo* treated immediately after death. For the muscle, 81–83% of the T.C.N. is soluble in LiCl and of this 65–71% occurs as myosin; of the salt-insoluble residue (17–19% of the T.C.N.) a further 5–10% is included in the acid or alkali extracts. The stroma fraction of 7–12% is considerably higher than the 3–5% reported by Reay & Kuchel [1936] for haddock muscle but there is as yet no systematic evidence that this fraction is constant in different muscles. The other figures presented here are in good agreement with the analyses of these authors.

Table I. *Distribution of N in g./100 g. wet tissue*

	Muscle			Electrical tissue						
	<i>T. mar- morata</i> (1)	<i>T. mar- morata</i> (2)	<i>T. ocel- lata</i> (3)	<i>T. mar- morata</i> (1)	<i>T. mar- morata</i> (2)	<i>T. ocel- lata</i> (3)	<i>R. clavata</i> *		<i>R. radiata</i> *	
Dry weight	23.2	23.8	26.2	10.8	10.6	11.8	10.4	10.0	12.0	12.8
Non-coagulable N	1.66	1.74	1.72	1.75	1.66	1.68	1.06	1.07	0.47	0.51
Total coagulable N	2.345	2.534	2.828	0.39	0.36	0.44	0.818	0.80	1.51	1.52
Coagulable N:										
In LiCl extract	1.89	2.11	2.34	0.25	0.22	0.314	0.333	0.30	0.65	0.67
In N/20 NaOH extract	2.09	—	—	0.27	0.227	0.34	—	—	—	—
In N/50 HCl extract	—	2.35	2.48	0.175	0.146	0.20	—	—	—	—
Myosin	1.67	1.65	1.90	Nil	Nil	Nil	Nil	Nil	0.15	0.09

\* T.C.N. of *R. clavata* muscle 2.63; *R. radiata* muscle 2.41. Dry weight of *R. clavata* muscle 23.4% *R. radiata* muscle 19.5%.

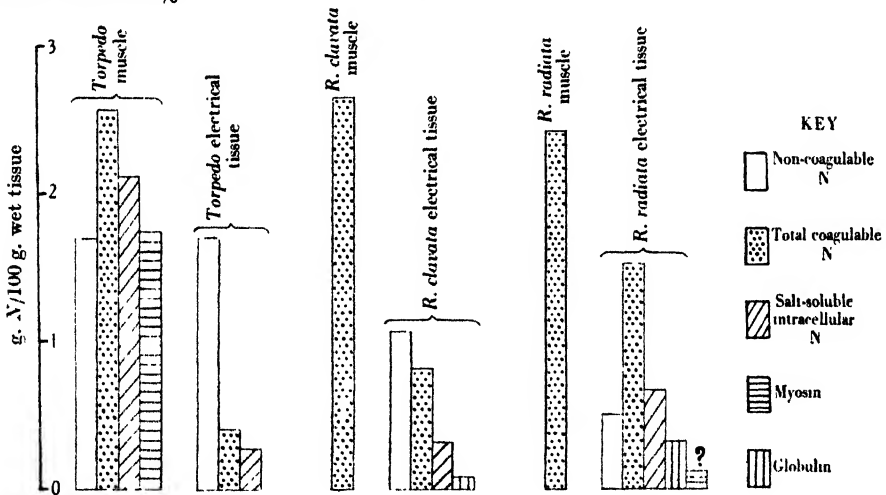


Fig. 1.

In comparison, it is seen that the dry weight of *Torpedo* electrical tissue is  $< \frac{1}{2}$  and the T.C.N. only  $\frac{1}{3} - \frac{1}{4}$  that of muscle and of this total protein, 61-77% is soluble in salt, 63-77% in N/20 NaOH and 40-45% in N/50 HCl. The comparatively large deviation in the amount of extractable coagulable N between individual organs of different specimens has been confirmed many times and may be explained by an observation of Ewart that the discs of connective tissue increase in diameter with age and decrease in thickness, whilst the amount of "gelatinous insulating material"—shown later to be a mucin—is considerably augmented; the proportion of intracellular N to stroma-N might therefore depend upon the age of the specimen. The figures reveal, however, that whereas in individual specimens, salt or alkali are equally effective in extracting the intracellular protein, the coagulable N extracted by acid is far lower, and in this respect the system differs fundamentally from muscle. For detection of myosin, it is not possible to utilize the sol obtained by exhaustive extraction of the tissue since the protein concentration is too low. More concentrated extracts obtained by extracting 1 part of tissue with 1 part of 14% LiCl have never yielded a precipitate. After 20-fold dilution with water, the faint opalescence which appears is due most probably to a small amount of apparent

globulin which precipitates when the LiCl sol is dialysed to a molarity of 0.03. This precipitate, which is small in comparison with the protein remaining in solution, is not a characteristic tissue globulin since it does not coagulate on heating.

The dry weight of the electrical tissue of *R. clavata* is about  $\frac{1}{2}$ , and the T.C.N. approximately  $\frac{1}{3}$  that of the corresponding muscle; only 37–41 % of the total protein is soluble in LiCl, and salt extracts containing 0.5 g. N/100 ml. showed only a faint turbidity when diluted with 20 vol. of water. Although myosin thus appears to be absent, a heavy globulin precipitate was obtained on dialysis to a salt molarity of 0.002. The protein remaining in solution did not precipitate when the salt concentration was further reduced. The amounts of globulin obtained from various LiCl extracts bear a constant ratio to the amount of coagulable N in the extract (Table II).

Table II

	(a)	(b)	(c)
Coagulable N extracted by LiCl as % of T.C.N. (A)	31.4	35.4	35.4
Coagulable N in globulin fraction as % of (A)	26.4	27.8	26.3

Mean coagulable N of globulin fraction = 26.8 % of the salt-soluble protein or 9 % of the T.C.N.

The comparatively large amount of salt-insoluble protein in this tissue is to be expected from the known existence of relatively dense septa of connective tissue separating the electric discs.

Experiments with the electrical tissue of *R. radiata* were greatly handicapped by the small size of the organ, 100 dissections yielding about 6.2 g. of wet tissue. The dry weight of the organ is somewhat higher than that of previous tissues and the T.C.N. now reaches  $\frac{2}{3}$  the value of *R. radiata* muscle. About 44 % of the T.C.N. is extractable by LiCl, and on dilution to a salt molarity of 0.07 a myosin-like precipitate amounting to 6–10 % of the T.C.N. separates. This precipitate, after centrifuging, was largely denatured and did not give the strong elastic film characteristic of myosin proper; moreover, the myosin prepared from *R. radiata* muscle did not denature under comparable conditions. Dialysis of the LiCl extract to lower salt concentrations (0.004 *M*) yielded an abundant globulin precipitate which was heat-coagulable. The total globulin precipitated on complete dialysis, determined in two separate extracts, amounted to 21 % of the T.C.N. and the amount of globulin salted out by saturation with NaCl amounted to a comparable figure of 19 %.

Thus of all the electrical tissues examined, that of *R. radiata* most closely resembles the protein system obtaining in muscle not only in the amount of T.C.N. but also in the comparatively large proportion of globulin, some of which precipitates at a salt molarity usually adopted for separation of myosin, but which has not been identified as myosin proper. When the results are represented diagrammatically (Fig. 1) the comparison between muscle and electrical tissue is seen to advantage. It may also be noted from this diagram that the non-coagulable nitrogenous constituents are highest where the T.C.N. is least, and in *Torpedo* amount to 1.7 % of the wet weight of the electrical organ.

*General properties of electrical tissue proteins.* The quantitative results indicate that all types of electric tissue contain a water-soluble protein; in *Torpedo* the whole of the salt-soluble protein is also water-soluble and in *R. clavata* and *R. radiata* a water-soluble fraction with properties similar to those of *Torpedo* protein is obtained after removal of globulins by complete dialysis. The dialysis liquors have a pH of approximately 7, and at this reaction the water-soluble protein is not coagulated by heat, is not immediately precipitated by  $\frac{1}{2}$  saturated

( $\text{NH}_4$ ) $_2$ SO $_4$ , saturated NaCl or by addition of 1-2 vol. of alcohol. The globulins on the other hand precipitate under these conditions and are heat-coagulable. The water-soluble fraction is precipitated at pH 4.5-5 and in this range it can be denatured by heating; at lower pH values it passes into solution and on neutralizing it again precipitates at the point of minimum solubility and re-dissolves at pH 7.5. In marked contrast to these properties the water-soluble fraction (myogen) of mammalian muscle, investigated by Smith [1937], and of *R. radiata* muscle, investigated by the author, is heat-coagulable, and although it denatures on acidification in absence of salt it does not precipitate until the solution is neutralized. The properties of the water-soluble proteins of electrical tissue are not unlike those of some mucins and nucleoproteins and the preparation of the solid protein from *Torpedo* was undertaken to characterize it more completely.

*Preparation of Torpedo protein.* The minced tissue was extracted with an equal volume of water or of 14% LiCl, stirred mechanically for 1 hr. and centrifuged. The extraction was once more repeated and the combined extracts were dialysed at 1° against distilled water. After centrifuging away a small amount of precipitate, the liquid was filtered through paper pulp, acidified to pH 4.5 and the supernatant was decanted from the protein precipitate which was now dissolved at pH 7.5, centrifuged, and precipitated as before. It was dried thoroughly in alcohol and ether, and a chance evaporation of these liquors revealed the presence of lipid which is discussed in a later section.

Two main samples of protein were analysed (Table III), *A* being prepared from a LiCl extract and *B* from a water extract.

Table III

Figures given are in percentages of total (ash free) dry wt.

	<i>A</i>	<i>B</i>
Ash	0.6	1.2
N	14.4	14.5
Amide-N	1.21	1.23
P	0.27	0.18
Carbohydrate	~ 6.5	~ 6
Glucosamine	3.5	3.8
Total S	1.38	1.33
SO $_4$ -sulphur split off on hydrolysis by HCl	0.47	0.49
Cystine-S (differential oxidation)	0.40	0.39
Methionine-S (by difference)	0.51	0.45
Tyrosine	3.9	—
Tryptophan	1.32	—

The methods adopted in this analysis are described elsewhere: amide-N, total S, tyrosine and tryptophan [Bailey, 1937, 1]; P [Fiske & Subbarow, 1925]; cystine-S by differential oxidation [Lugg, 1938]; carbohydrate, the method of Tillmans & Philippi [1929] adapted by Pirie [1936]; glucosamine, the Zuckerkandl & Messiner-Klebermass method [1931] modified by Hewitt [1938].

The presence of carbohydrate, glucosamine, and of ethereal sulphate split off after hydrolysis with 5N HCl at 100° for 12 hr., confirms the inference, derived from physico-chemical tests, that the protein belongs to the class of mucins. The P appears also to be an integral part of the protein; it is not split off by dilute N/70 HCl in the cold, and only 26% is freed by digestion with N NaOH at 100° for 5 min. A P-rich residue was obtained in the following way: 1.58 g. of protein (P, 0.3%) were digested with 10 ml. of 0.5% pepsin solution in 30 ml. N/70 HCl at 27° for 2 days. The undigested sediment after washing

and drying amounted to 23 % of the original protein and gave on analysis: ash, 1.0; N, 13.6; P, 0.8; S, 1.6 %. Whether this residue is a nuclein cannot categorically be affirmed since attempts to isolate nucleic acid from the amount of material available have failed.

Similar proteins have been described in the literature; eel-slime, investigated by Müller & Reinbach [1914], has similar physico-chemical properties and gives a comparable analysis (N, 14.0–14.4; S, 1.16–1.29; P, 0.33–0.49 %) and in its natural state is associated with lipid. The “nucleoalbumin” isolated by Hammarsten [1885] from snail liver possesses similar properties, giving on analysis, N, 14.3; S, 1.06; P, 0.42 %; when digested with pepsin a nuclein containing 2.1 % P was obtained. In *Torpedo* extracts it was at first suspected that the P might arise from the abundant nuclei of the electrical layer, the isolated protein thus consisting of a mixture of mucin and nucleoprotein. Since, however, the P occurs in Müller & Reinbach’s protein, which is an external skin secretion, it is unlikely that such admixture is possible, and although the P reacts as nucleic acid-P, its exact assignation to a definite prosthetic group must await further research.

*Isoelectric points of electrical tissue proteins.* These were determined by the cataphoretic method of Dummett & Bowden [1933] using 0.01 *M* phthalate buffers. Dried and undried preparations of proteins were ground with a little water and lightly centrifuged; aliquots of the particle suspension in the supernatant were added to a buffer series and the approximate velocity of migration was plotted against the *pH* value of the solution, measured after completion of the experiment by the hydrogen electrode. The values are given in Table IV.

Table IV

	Isoelectric point
1. <i>Torpedo</i> mucin, dried preparation, fat-free	3.15
2. <i>Torpedo</i> mucin + fat, undried	3.1
3. <i>R. clavata</i> mucin, dried preparation, fat-free	3.5
4. <i>R. clavata</i> mucin + fat, undried	3.6
5. <i>R. clavata</i> globulin, dried, fat-free	4.1
6. <i>R. clavata</i> globulin + fat, undried	4.1
7. <i>R. radiata</i> mucin + fat, undried	3.55
8. <i>R. radiata</i> mucin + fat, undried	3.6
9. <i>R. radiata</i> globulin, dried, fat-free	4.1
10. <i>R. radiata</i> myosin (from muscle)	4.6

*Note on the preparations listed in Table IV.* The globulin fractions 5 and 6 were obtained by dialysis of the tissue extracts and were washed thoroughly with water; their purification was not undertaken as they were partly denatured; globulin 9 was obtained by saturation of the LiCl extracts with NaCl. The mucins 3 and 4 were prepared after removal of globulins by dialysis, the residual liquors being filtered through paper pulp and acidified; the precipitated protein was then purified by redissolving at *pH* 7.5 and reprecipitating twice. The mucin of *R. radiata* was more difficult to prepare, owing to contamination of the dialysate with colloidal globulin; preparation 7 was obtained by saturating the dialysate of the LiCl extract with NaCl when a small amount of globulin separated and, after dialysis of the residual liquor, the protein was precipitated by acidification; preparation 8 was obtained by saturating the LiCl extract with NaCl and, after removal of the globulin by centrifuging and of NaCl by dialysis, the residual liquor on boiling gave a slight coagulum, which was filtered off and the mucin precipitated by acidification. The myosin (10) was prepared by the Edsall [1930] method and twice precipitated from salt solution.

Within 0.1–0.2 pH unit from the designated isoelectric point, differences in the direction of migration could sometimes be observed only in the case of the globulin fractions, indicating, as indeed is most likely, some degree of non-homogeneity. On the other hand, the isoelectric point of the mucin fractions was very sharp, and the low values obtained confirms their assignation to this group of proteins. The globulin fractions in bulk have an isoelectric point lower than that of *R. radiata* myosin, which in turn is lower than the reported values for that of mammalian muscle. The isoelectric point of the protein-lipid complex does not differ greatly from that of the alcohol-extracted protein.

*The lipid material associated with electrical tissue proteins.* All globulin and mucin fractions prepared from salt or water extracts of electrical tissue contain considerable amounts of lipid which can be separated by extraction with hot alcohol and ether. After evaporation of the extracts, the residue was freed from traces of protein by re-extracting with chloroform. The amount of lipid associated with various preparations of protein was investigated only in the case of *Torpedo* mucin, and this was not constant; e.g. the lipid contents as % of six lipid-protein preparations were 15.2, 14.0, 22.4, 23.0, 23.0, 14.9 % respectively.

The combined lipid fractions from several preparations of *Torpedo* mucin were combined, re-extracted with chloroform, evaporated and dried at 100° to constant weight. The material contained 1.92 % P and 1.4 % N. The free cholesterol was estimated in the usual way by precipitation with digitonin, and the cholesterol as ester, after saponification with *N* sodium ethoxide and removal of unsaponifiable material. The free fatty acids were next extracted and weighed and the contents of phosphatides and glycerides present in the original lipid fractions were then calculated on the assumption that the P was due exclusively to lecithin, and the fatty acids of both to oleic acid. The following figures were thus obtained:

Phosphatide	...	...	...	...	...	50.6 %
Free cholesterol	...	...	...	...	...	19.4 %
Unsaponifiable matter other than cholesterol	...	...	...	...	...	10.0 %
Glycerides	...	...	...	...	...	7.1 %
Total	...	...	...	...	...	87.1 %

The amount of cholesterol ester was insignificant.

It is of immediate chemical interest to consider the function of lipid in its association with mucin, since this combination, found also in eel-slime, is probably of general occurrence in the fish mucins. It appears to act as a dispersing agent for the protein since its removal by alcohol extraction leads to complete insolubility of the protein above and below its isoelectric point, and although the denaturing action might be ascribed to the use of organic solvents, it is significant that the original protein complex is not affected by the stronger denaturing actions of heat and acid. In its native state the complex has the solubility characteristics of the mucoids, but this term has been avoided because it has not been possible to assess how much the lipid fraction contributes to the solubility of the protein *per se*. Although the mucin-lipid complex is stable in neutral solution, those of the globulins lose their solubility in salt after dialysis, and the globulin-LiCl extracts denature slowly on standing. These phenomena are probably due to the denaturing action of phosphate acting in the manner described by Hardy & Gardiner [1910] for the plasma proteins and resulting, in the view of Chick [1914] in the production of euglobulin from pseudoglobulin.

## SUMMARY

1. Since the electrical and muscular tissues of elasmobranchs arise from the same embryonic rudiments, a comparative study of the protein systems of the electrical tissue of *Torpedo*, *Raia clavata* and *R. radiata* has been made to ascertain whether such systems are comparable with those of muscle.

2. The N-partition of *Torpedo* muscle and of the three types of electrical tissue has been determined. Quantitatively, electrical tissue is characterized by a low total solid and low protein content; the amount of total coagulable N is highest in *R. radiata*, decreasing progressively in *R. clavata* and *Torpedo*, and the proportion of this N existing as globulin decreases in the same way. In *Torpedo* the extractable protein is almost entirely a mucin and this is common to the other electrical tissues examined.

3. The cataphoretic isoelectric points of the globulin fractions and the mucins have been determined.

4. All protein fractions are associated with comparatively large amounts of lipid, which was isolated from *Torpedo* mucin and analysed. It consists mainly of phosphatide, free cholesterol and glyceride.

5. A partial analysis of *Torpedo* mucin is presented. It is shown to contain ethereal sulphate, carbohydrate and glucosamine, and non-labile P, which is obtained in a P-rich residue after digestion with pepsin. Attempts to isolate nucleic acid have failed.

6. The results support the view that electrical tissue, whilst retaining the enzymes associated with the glycolytic system of skeletal muscle, differs mainly in the absence of myosin, which is no longer necessary for contraction. The protein system is most dissimilar from muscle in those tissues which, from the histological standpoint, are farthest removed from muscle (e.g. *Torpedo*) and most similar in incompletely metamorphosed tissue (e.g. *R. radiata*).

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# XXXII. A COLORIMETRIC REACTION FOR THE QUANTITATIVE ESTIMATION OF NICOTINIC ACID

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Now that it is known that nicotinamide is a component of cozymase, and that nicotinic acid possesses a curative effect on pellagra, a method by which this substance could be determined qualitatively and quantitatively, and which would be suitable for serial work, would be valuable.

Methods of analysis have been published previously by Swaminathan [1938], Karrer & Keller [1938], Vilter *et al.* [1938] and quite recently by Shaw & Macdonald [1938]. None of these methods is based on a specific reaction with nicotinic acid; they are founded on a colorimetric reaction of the pyridine ring.

Swaminathan's and Shaw & Macdonald's methods are based on the observation made by König [1904], that pyridine with cyanogen bromide and a primary or secondary aromatic amine develops a colour, which, according to the amine employed, varies from yellow to violet. The chemical reactions leading to colour formation have not yet been fully elucidated. Kulikow & Kresdowosdwigenskaja [1930] worked out a method based on this for determining small amounts of pyridine using aniline. Karrer & Keller [1938] and Vilter *et al.* [1938] based their work on the observation of Vongerichten [1899] who found that a colour reaction ensues when pyridine reacts with 2:4-dinitrochlorobenzene and alkali hydroxide.

Up to the present none of these methods has proved quite satisfactory. Swaminathan [1938], who as mentioned used aniline, extracted the resulting colour by shaking with *isoamyl* alcohol. In our hands this method has yielded unsatisfactory results; the colour developed is inconstant and it is difficult to obtain pyridine-free *isoamyl* alcohol.

We have not had the opportunity to test Shaw & Macdonald's [1938] method of analysis, as this appeared after we had completed the experimental part of this work. The authors, however, state that the intensity of the colour is not constant at any time, which renders their method unsuitable at any rate for serial analyses. The methods described by Karrer & Keller [1938], and by Vilter *et al.* [1938], appear to be laborious and tedious, and the authors mention that the colour obtained is very unstable.

In the present work, an account is given of a colour reaction based on König's principle. It is rapid to carry out, is performed in aqueous solution and gives a constant colour.

As colour-producing amine we have tried Na sulphanilate, Rodinal (*p*-aminophenol) and sulphanilamide (*p*-aminobenzenesulphonamide), which however gave inconstant results, whereas we found that metol (*p*-methylaminophenol sulphate) with nicotinic acid and CNBr in aqueous solution yields a clear yellow colour which, under the conditions used by us, is perfectly constant, very stable and of an intensity directly proportional to the amount of nicotinic acid.

## I. DETERMINATION OF NICOTINIC ACID IN COLOURLESS AQUEOUS SOLUTION

*Technique*

A measured amount (up to 9 ml.) of the aqueous solution of nicotinic acid to be analysed (containing from 0.005 to 0.25 mg. nicotinic acid) is run into a graduated 20 ml. flask. After 5 min. heating on a water bath at 75–80°, 1 ml. 4 % aqueous CNBr is added. The mixture is placed on the water bath for 5 min. and then cooled under the tap to room temperature. 10 ml. saturated aqueous metol (about 5 %), and distilled water to make up the volume to 20 ml., are added. After standing 1 hr. at room temperature excluded from light, the strength of the colour developed is read off with a Pulfrich photometer (filter S. 43), with a blank solution containing the same amounts of CNBr and metol plus distilled water to make the volume 20 ml. in the other cell.

A. *The reagents*

1. *Cyanogen bromide*. We have used CNBr supplied by Fraenkel & Landau, Berlin-Oberschöneweise. A 4 % aqueous solution is employed, and it must be prepared on the day of use.

A solution of CNBr prepared as described by Kulikow & Kresdowosdwigenskaja [1930] may also be used. To a saturated aqueous solution of Br, KCN is added just to decoloration. This solution contains about 5 % CNBr.

2. *Metol* (*p*-methylaminophenol sulphate);  $(\text{CH}_3\text{NHC}_6\text{H}_4\text{OH})_2, \text{H}_2\text{SO}_4$ . The metol employed is the usual product supplied by Agfa. An aqueous solution, saturated at room temperature, i.e. c. 5 %, is used. Thorough shaking is necessary as the metol dissolves rather slowly and the solution must not be heated. It is protected from light and must be used within 2–3 hr. of preparation. Either on heating or on further standing the solution turns brownish-violet.

B. *The analysis*1. *The reaction between nicotinic acid and CNBr*

(a) *Influence of temperature*. At room temperature the reaction between nicotinic acid and CNBr proceeds very slowly. At 75–80° the reaction takes place so quickly as to be completed in about 3 min. If the mixture is heated to this temperature for more than 20 min. the compound first formed slowly disintegrates.

(b) *The amount of CNBr*. At least 30 mg. CNBr must be used for amounts of nicotinic acid up to 0.25 mg. Larger quantities (up to 80 mg.) do not change the extinction constant; but smaller quantities give too low an extinction value.

2. *The reaction with metol of the compound formed by nicotinic acid and CNBr*

(a) *Amount of metol*. The extinction constant increases with the concentration of metol in the solution until a certain point is reached. For metol quantities of 500–650 mg. in a volume of 20 ml. (i.e. from 10 to 13 ml. 5 % solution) the extinction is constant (see Fig. 1). The quantity of metol determined in this way appears rather large in relation to the amount of nicotinic acid. On recrystallization of the metol exactly the same results were obtained; thus the colour reaction can hardly be ascribed to impurities in the reagent. The final intensity of the colour depends on the metol concentration and not on the absolute quantity of metol: with a certain amount of nicotinic acid plus 250 mg.

metol diluted to a total volume of 10 ml. the colour intensity will be exactly twice that resulting from the same amount of nicotinic acid plus 500 mg. metol in a volume of 20 ml.

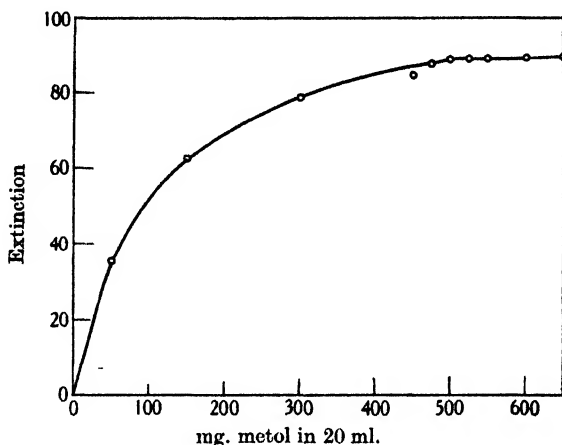


Fig. 1. Extinction obtained from 0.1 mg. nicotinic acid with 1 ml. 4 % CNBr and varying amounts of metol in a volume of 20 ml.

(b) *Time of reaction with metol.* On addition of metol a faint yellow coloration appears immediately and quickly intensifies. The intensity of the colour, however, first reaches its maximum after  $\frac{1}{2}$ –1 hr.

(c) *Constancy of the colour developed.* At room temperature with protection from light the colour of the solution remains unchanged after 72 hr.

(d) *The effect of light.* If, after metol has been added, the mixture is kept in full daylight, the colour developed is fainter than if the solution has been kept in darkness; continuous exposure will result in gradual fading. However, the sensitiveness to light is not so great that the colour fades appreciably in diffuse daylight in 5–10 min.

(e) *The temperature.* No change occurs in the colour produced if the solution, after adding metol, is kept for 1 hr. at temperatures ranging from 5 to 30°. At 75–80° the colour developed is considerably fainter, beginning as a clear yellow it subsequently, after 20–30 min., turns more brownish-red.

### 3. Photometric determination

(a) *Colour.* The colour developed is a clear yellow. The extinction corresponding to the various filters of the Pulfrich photometer are approximately (0.1 mg. nicotinic acid in 1 ml. layer): S. 43, 0.89; S. 45, 0.48; S. 47, 0.26; S. 50, 0.05; S. 53–S. 75, 0.00.

(b) *Measurement.* With cells of 2.5–50 mm., it is possible to determine nicotinic acid in quantities ranging from 0.005 to 0.25 mg. Larger quantities yield an extinction which is too high to be measurable in a layer of 2.5 mm. In case the colour is too intense, diluting the solution with water before measurement is without avail, as this lowers the concentration of metol, thus changing the extinction constant. A fresh analysis must be performed on a more diluted solution of the sample to be examined.

(c) *Blank.* The blank is made with distilled water in place of the nicotinic acid solution. Blanks give a very pale pink colour. It has been observed that

CNBr preserves metol from changing to the coloured substances mentioned above; even after standing for several days in full daylight the colour of the blank does not change perceptibly.

Using the filter (S. 43) employed in the analysis in a layer of 1 cm. the blank analysis with distilled water gives an extinction which, reckoned from the moment of adding metol to 24 hr. later, gradually increases from 0.02 to 0.04.

#### 4. Determination of the extinction curve

With known amounts of nicotinic acid the extinctions given in Fig. 2 have been found. Within the limits of the quantities measured the colour developed is directly proportional to the amount of nicotinic acid.

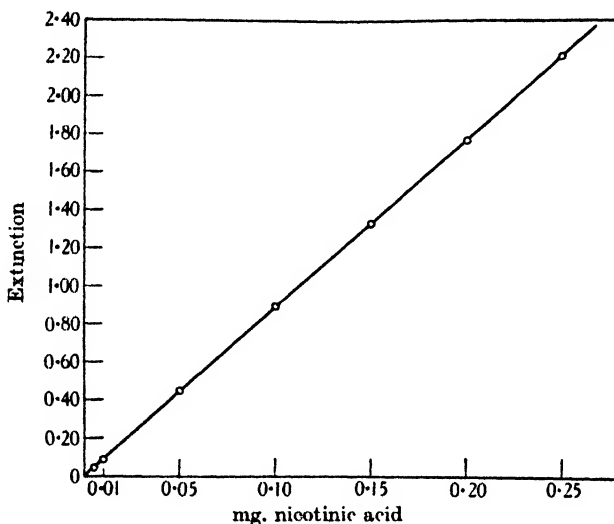


Fig. 2. Calibration curve of nicotinic acid.

Under the conditions mentioned, we have not found any variation in the extinction constant; nevertheless, as a control in each series of analyses, we have determined the value of a standard solution with known content of nicotinic acid (0.1 mg.). This has, in addition, served as a control on the reagents.

For working out the standard analysis 1 ml. of a 1:10,000 solution of nicotinic acid may suitably be used. In this feeble concentration the nicotinic acid is stable for a few days only. In a concentration of 1:1000, and in a brown glass receptacle, nicotinic acid is stable for several months at least.

#### 5. The sensitiveness and accuracy of the method

As mentioned, quantities of nicotinic acid as small as 0.005 mg. may be determined with great accuracy.

It is difficult to give the percentage error. After numerous tests we believe that when pure aqueous solutions of nicotinic acid are used the error of the method is derived chiefly from the error in the photometric readings.

The method of analysis described has been worked out with pure aqueous solutions of nicotinic acid. An equiv. wt. of 122.4 (calculated 123.05) was found for the nicotinic acid used (J. D. Riedel-E. de Haën, A. G.); m.p. 232°, as stated in the literature.

The colour reaction does not tolerate free strong acid or base. In the presence of acetate ions a red-brown colour develops. Sulphate (in higher concentration than it occurs in metol) and nitrate ions cause no change in the tint, but weaken the intensity of the colour.

NaCl,  $\text{NH}_4\text{Cl}$  and  $\text{KH}_2\text{PO}_4$  cause no change in either the tint or intensity of the colour.

If the reaction occurs in presence of acetone or alcohol, the reading is lower and inconstant.

#### *Nicotinamide*

If nicotinamide is examined by the process described above, the same results are not obtained as for equivalent amounts of nicotinic acid. The same clear yellow colour is produced, but it is usually considerably stronger, and the results are not reproducible. Therefore if nicotinic acid occurs partly or wholly as amide, it must be hydrolysed before the analysis.

## II. DETERMINATION OF NICOTINIC ACID IN ORGANIC MATERIAL

In biological material, nicotinic acid occurs—at any rate to a large extent—in the form of the amide as a component of cozymase or Warburg's coferment: prior to determination it must, therefore, be liberated and the nicotinamide hydrolysed. We have found that this can be accomplished by heating with NaOH: as it appeared that the same result was obtained by heating on a boiling water bath for  $\frac{1}{2}$  hr. as by heating in the autoclave for periods up to 2 hr. at  $120^\circ$ , followed by heating with  $2N$  HCl (Table I), the first procedure was adopted.

Table I

Treatment	Dried yeast. Nicotinic acid found mg. %	Dried yeast + 50 mg. nicotinic acid (as amide) per 100 g. Total nicotinic acid found mg. %	Added nicotinamide found (as nicotinic acid) %
On boiling water bath for:			
30 min.	53.2	—	—
60 min.	54.2	105.7	103
90 min.	53.4	—	—
120 min.	53.2	—	—
Heating in an autoclave at $120^\circ$ for:			
30 min.	53.2	102.5	99
60 min.	53.0	103.5	101
120 min.	53.2	97.6	89

Nicotinic acid added to the samples was in every case recovered quantitatively by these methods. This process results in a highly coloured homogeneous mass, which must be rendered quite colourless before it is suitable for colorimetric determination.

The removal of the colour by adsorption with charcoal or Fuller's earth at different reactions was unsuccessful, as the nicotinic acid is adsorbed with the coloured substances, and cannot be entirely eluted separately. As even filter paper adsorbs nicotinic acid, filtering must not take place at any stage of the analysis. Nor was precipitation of the coloured substances with heavy metal salts and subsequent removal of the metal ions with  $\text{H}_2\text{S}$  satisfactory, while  $\text{HgCl}_2$  and  $\text{Ag}_2\text{SO}_4$  did not precipitate the coloured substances. Pb acetate and  $\text{Hg}(\text{NO}_3)_2$  cannot be used as the anions of these salts interfere with the colour

reaction. The best result, although still not satisfactory, was obtained by precipitation with  $\text{CuCl}_2$ . Evaporation to dryness and subsequent extraction with benzene and acetone were discarded, as neither the preformed nor the added nicotinic acid could be extracted quantitatively after drying.

However, it was found that the coloured substances could be precipitated from an aqueous phase by a large excess of acetone, in which nicotinic acid is relatively easily soluble. We have employed this procedure to remove the colour. Based on these experiments, we have worked out a method of analysis. As an example, the technique used in examining dry yeast will be described in the following.

### *Technique*

10 ml. 2*N* NaOH are run into a 20 ml. graduated flask without moistening its neck, and 5 g. of dry yeast are added (the order must not be reversed as the yeast will then collect in lumps which will merely be attacked superficially). The neck of the flask is closed with a wad of non-absorbent cotton wool. After shaking, the flask is placed on a boiling water bath for 30 min.; the yeast will then be fully dissolved. After cooling, concentrated 36% HCl (1.8 ml.) is added drop by drop, and the mixture thoroughly shaken. The reaction will now have become slightly acid ( $\text{pH} \approx 4-6$ ). The solution is cooled to about  $20^\circ$ , and distilled water is added to make up the volume to 20 ml.

The contents are thoroughly mixed and allowed to stand for several minutes. Part is then centrifuged in a 15 ml. centrifuge tube. A voluminous sediment (amounting to about one-half to one-third of the total) is obtained; the upper layer consists of a slightly turbid dark brown liquid.

Exactly 1 ml. of the centrifugate (corresponding to 250 mg. yeast) is placed in a second centrifuge tube, which is well shaken while exactly 9 ml. acetone are added slowly from a burette. The tube is carefully closed with a rubber stopper, and after vigorous shaking, it is centrifuged for 3-4 min. The contents will then have separated into two layers—a very small (about 0.3 ml.) intensely coloured very viscid aqueous phase, and a clear practically colourless layer of aqueous acetone.

3 ml. (corresponding to 75 mg. yeast) of the acetone layer and 3 ml. distilled water are then mixed in a round-bottomed flask and the acetone evaporated with a water vacuum pump, without other heating than the warmth of the hand. After evaporation of the acetone, the contents are quantitatively transferred with the aid of  $N/15 \text{ KH}_2\text{PO}_4$  (which ensures that the reaction does not become too acid) to a graduated flask of 20 ml. capacity. The volume must not exceed 9 ml.

The colour reaction described above is carried out on the practically clear colourless solution thus obtained and, with the aid of the coefficient of extinction controlled by the standard solution (0.1 mg. of nicotinic acid), the amount of nicotinic acid contained in the sample of yeast is determined.

### *Comments*

After treatment with NaOH, the yeast becomes a homogeneous mass; a voluminous precipitate appears on cooling and particularly on neutralizing with hydrochloric acid. The mixture is cleared by centrifuging and aliquot parts are employed for the extraction with acetone. That this is allowable is shown by the fact that the intensity of the colour produced is the same whether a part of the clear centrifugate or a part of the total mixture is used. However, it is

advisable to remove the precipitate by centrifuging; otherwise, on transference of the acetone extract to water, a turbidity will appear, which—although diminishing considerably during the colour reaction—nevertheless complicates the photometric determination. The error in the readings may amount to 5% at most; this error is avoided by removing the precipitate.

With regard to the acetone extract the following point must be mentioned: if too large an excess of acetone is used, a fairly solid resinous precipitate is formed, containing a relatively large part of the nicotinic acid (about 30%). On the other hand, too small an amount of acetone will not completely precipitate the coloured substances.

The principle to be observed in extraction is to use exactly that quantity of acetone which gives a sufficiently colourless and clear solution without leaving a precipitate that is too solid.

In the case of yeast this is achieved by using the quantities described. The precipitate amounts to about 0.3 ml., and consists partly of solid flakes, and partly of a very viscid brown liquid. Analysis of the precipitate (dissolved in 0.7 ml. distilled water and again extracted with acetone) has shown that the concentration of nicotinic acid is the same as in the acetone phase.

The transference of the acetone extract to water is rendered necessary by the fact that the presence of even small quantities of acetone during the process of heating with CNBr will result in lower values.

It is essential to evaporate the acetone at low temperature; high temperature, e.g. heating on a boiling water bath, will cause some of the nicotinic acid to volatilize with the acetone. Control analysis with known quantities have shown that the nicotinic acid is recovered quantitatively when the acetone is evaporated in vacuum with no other heating than the warmth of the hand. By evaporation on a boiling water bath of an aqueous solution of nicotinic acid (containing about 0.1 mg. of nicotinic acid) the loss is quite large; it is least when the solution contains NaOH, but very considerable if the solution is made alkaline with  $\text{NH}_3$  or acid with a mineral acid. Presence of acetone during evaporation increases the loss of nicotinic acid.

The analysis of various kinds of yeast has given the results shown in Table II.

Table II

	Nicotinic acid in dry matter mg. %
Tuborg 140 Surface-yeast	45.5
168 Surface-yeast	44.6
113 Bottom-yeast	36.2
173 Bottom-yeast	33.9
175 Bottom-yeast	36.7
7 Wine-yeast	17.7
11 Wine-yeast	15.9
Fæx medicinalis I	61.2
II	57.2
III	55.9
Compressed baker's yeast	25.7

## SUMMARY

An exact colorimetric method of analysis for determining nicotinic acid in aqueous solution has been elaborated.

The method depends on the addition of cyanogen bromide at 70–80° and the production of a colour with metol (*p*-methylaminophenol sulphate) at room temperature.

The method has been employed to analyse samples of yeast. From 16 to 61 mg. of nicotinic acid % dry wt. have been found in the various types of yeast.

We wish to thank the Tuborg Breweries for submitting the yeast samples.

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# XXXIII. POLYSACCHARIDES SYNTHESIZED BY MICRO-ORGANISMS

## IV. THE MOLECULAR CONSTITUTION OF LUTEOSE

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*(Received 29 December 1938)*

LUTEIC acid, a highly mucilaginous polysaccharide, is a metabolic product of *Penicillium luteum* Zukal when this mould is grown on liquid Czapek-Dox medium with any one of the following compounds as the sole source of carbon: glucose [Raistrick & Rintoul, 1931], fructose, galactose, mannose, xylose, arabinose and glycerol [Birkinshaw & Raistrick, 1933]. Raistrick & Rintoul [1931] showed that on acid hydrolysis luteic acid yielded malonic acid (1 mol) and glucose (2 mols) and that it was probably a malonyl ester of a polyglucose. Mild alkaline hydrolysis of luteic acid eliminated the malonyl groups as malonic acid and produced a neutral polysaccharide which, when hydrolysed, yielded glucose alone, and to which the name "luteose" was given. In the present paper an investigation of the molecular structure of luteose is recorded.

The experimental work has been carried out on luteic acid produced by two different strains of *P. luteum* Zukal and the luteose samples prepared from each were examined separately. The first specimen of luteose (C. G. Anderson's preparation) was prepared from luteic acid according to the method described by Raistrick & Rintoul [1931]. It was a white powder, slightly soluble in water, mean  $[\alpha]_D -33^\circ$ . Alcohol-water fractionation revealed no essential differences in the optical properties of fractions but this fact cannot be taken as sole criterion of homogeneity. Luteose was acetylated by treatment with acetic anhydride in pyridine, followed by acetic anhydride containing sulphur dioxide and chlorine as catalysts according to Haworth & Machemer's [1932] modification of Barnett's method. Fractional precipitation of the product from chloroform solution by light petroleum gave fractions with identical optical properties and with an average acetyl content of 45.4%. The acetyl luteose was simultaneously deacetylated and methylated with dimethyl sulphate and 40% KOH in the presence of acetone by the method of Haworth *et al.* [1931]. Continued treatment raised the methoxyl content to 42.5% beyond which no increase occurred. Exhaustive extraction of this compound with acetone gave methylated luteose in 90% yield while the acetone extracts contained a methylated polysaccharide, OMe, 44.3%, which will be described later. Methylated luteose was a white powder  $[\alpha]_D -32^\circ$ , OMe, 41.5%, insoluble in acetone or ethyl alcohol. Its hydrolysis was accomplished by contact with cold fuming HCl during 156 hr. A preliminary separation of the liberated methylated sugars was made by extracting the neutralized aqueous solution with chloroform and the fractions

were separately converted into the glucosides by boiling with methyl alcoholic-HCl. The glucosides were fractionated by distillation in high vacuum and in this way it was possible to isolate 2:3:4-trimethyl methylglucoside (approx. 80 %) and a dimethyl methylglucoside (15 %). No tetramethyl methylglucoside was detected.

The investigation was repeated on a second sample of luteose (M. Stacey's preparation). This material was prepared from the luteic acid produced when a new strain of *P. luteum* Zukal was grown on glucose under the usual conditions. The metabolism solution was evaporated to small bulk, acidified to Congo red with HCl, the polysaccharide precipitated with alcohol and isolated in the usual way. On hydrolysis of the crude luteic acid with  $N$   $H_2SO_4$ , crystalline glucose (40 %), malonic acid (15 %) and a syrup (45 %) were obtained. The luteic acid gave less viscous solutions in water than the early specimens and, following the method of Raistrick & Rintoul, a 50 % yield only of luteose was obtained. It had  $[\alpha]_D - 32^\circ$  (in sodium hydroxide) and could be separated into water-soluble and water-insoluble fractions. A reliable rapid method of isolating luteose in purified form was developed by making use of the fact that on the addition of Fehling's solution to luteic acid the malonyl groups were removed and luteose formed an insoluble copper complex. Luteose was obtained as an ash-free white granular powder, insoluble in cold water,  $[\alpha]_D - 38^\circ$  (in sodium hydroxide), and yielding only glucose on acid hydrolysis. The luteose was methylated directly with dimethyl sulphate and 30 % NaOH in the presence of dioxane and recourse was not had to the intermediate formation of the acetate. Twelve methylations gave a methylated derivative having OMe, 43.6 %. Attempts to increase the methoxyl content by modification of the method of methylation were unsuccessful. Methylated luteose was fractionated in the usual way and viscosity determinations on the fractions revealed its essential homogeneity.

The operations of hydrolysis of the methylated luteose with fuming HCl, conversion of the product into the glucosides and distillation of the latter, were carried out as previously described. This separation gave approximately 85 % of trimethyl methylglucoside ( $\alpha$ - and  $\beta$ -forms) which partially crystallized, and approximately 10 % of a dimethyl methylglucoside. Despite careful search, no indications of the presence of a tetramethyl methylglucoside fraction could be obtained.

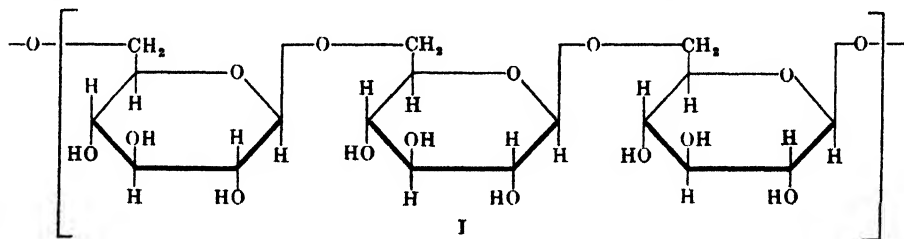
The trimethyl methylglucoside (crystalline fraction) was identified as 2:3:4-trimethyl  $\beta$ -methylglucopyranoside, M.P. 93–94°, since it showed no depression of M.P. in admixture with an authentic specimen and gave on hydrolysis 2:3:4-trimethyl glucose ( $[\alpha]_D + 60^\circ$  in water), identified as the anilide, M.P. 145–146°. The trimethyl methylglucoside (non-crystalline fraction) was shown to be a mixture of the  $\alpha$ - and  $\beta$ -forms of 2:3:4-trimethyl methylglucopyranoside in the following way. On hydrolysis 2:3:4-trimethyl glucose ( $[\alpha]_D + 60^\circ$  in water) was isolated and yielded the anilide, M.P. 145–146° described above. 2:3:4-Trimethyl glucose was oxidized with bromine water to give 2:3:4-trimethyl  $\delta$ -gluconolactone ( $[\alpha]_D + 82^\circ \rightarrow + 33^\circ$  in 26 hr.). The latter was further oxidized with  $HNO_3$  to 2:3:4-trimethyl saccharic acid which was isolated as the lactone methyl ester (M.P. 106–107° alone or in admixture with an authentic specimen). Part of the dimethyl fraction was methylated further to form tetramethyl methylglucoside which was hydrolysed to crystalline tetramethyl glucopyranose (80 % yield). The remainder was hydrolysed with 6 % HCl ( $[\alpha]_D^{20} + 95^\circ \rightarrow + 70^\circ$ ) and a syrupy dimethyl glucose, OMe, 28.4°,  $[\alpha]_D + 72^\circ$  was isolated. This was converted into the anilide, M.P. 120–130°, OMe, 20.3 %, which appeared to be a mixture and of which a satisfactory separation was not obtained.

Determination of the particle weight of methylated luteose by osmotic pressure measurements [Carter & Record, 1936] gave a value corresponding to about 80 glucose units.

Examination of the acetone extracts of methylated luteose (C. G. Anderson's preparation) revealed the presence of small amounts of a methylated polysaccharide, OMe 44.3 %, which yielded mannose derivatives on acid hydrolysis. In this connexion Anderson & Raistrick [1936] have shown that *P. luteum* Zukal, when grown on glucose under certain conditions, produced luteic acid together with small amounts of polysaccharides containing mannose, galactose or fructose units. A search was made for such compounds in the mother liquors from the purification of luteose with Fehling's solution. A polysaccharide containing galactose and mannose residues was isolated and its investigation will form the subject of a latter communication.

### DISCUSSION

The isolation of 2:3:4-trimethyl glucose in 85 % yield from methylated luteose clearly indicates that the basal constituent of the polysaccharide is a chain of glucopyranose residues united by 1:6-glucosidic linkages. Furthermore, it would appear from consideration of the optical rotation of luteose ( $[\alpha]_D - 32^\circ$ ) and the change in sign of rotation on hydrolysis ( $\rightarrow +18^\circ$ ) that the glucosidic linkages are predominantly of the  $\beta$ -type (I). Polysaccharides of similar



structure have been investigated here [Peat *et al.* 1938; Daker & Stacey, 1938] and elsewhere [Fowler *et al.* 1937]. All are of microbiological origin (the products of metabolism of the *Leuconostoc* species and of *Betabact. vermiforme* etc.) and are classed together as "dextrans". Special interest attaches to luteose in that it is the first of the dextrans investigated in which the type of linkage is  $\beta$ -glucosidic.

The apparent absence of an end-group (tetramethyl glucose) in methylated luteose is explicable on the assumptions either that the number of glucose units in the chain is too great to allow of the isolation of an end-group in sufficient quantity to be detectable under the conditions employed or, alternatively, that the chain exists in the form of a continuous loop. In connexion with the first hypothesis, the particle weight determined osmotically becomes of significance in that it shows the molecule to be finite in size and, indeed, not exceeding an aggregate of 80 hexose units. It is to be noted that the limits of experimental error of our method are such that the tetramethyl glucose derived from a polysaccharide constituted as an open chain of 80 units would easily have been detectable. The alternative of a closed chain structure must therefore be envisaged.

The unusually large proportion (10 %) of dimethyl glucose liberated together with the trimethyl glucose may also prove of importance when the finer structure

of the luteose molecule comes to be considered. The dimethyl glucose may arise from the presence of aggregating bonds or cross-linkages between relatively short chains of glucose units or it may simply be an expression of the observed resistance of the polysaccharide to the methylating reagents. Such resistance may be of course of a purely mechanical kind, dependent upon the physical condition of the polysaccharide during methylation and in support of this view the difficult solubility of methylated luteose in the usual solvents may be instanced. The data at present available, however, are insufficient to allow a decision to be made on this point.

#### EXPERIMENTAL

##### *Luteose. Sample I (C. G. Anderson's preparation)*

Luteic acid (112 g.) prepared from cultures of *Penicillium luteum* Zukal, Cat. No. Ad. 30 and purified according to Raistrick & Rintoul [1931] was dissolved in water (1200 ml.), *N* NaOH (178 ml.) was added, and the solution kept overnight at room temperature. The excess alkali was neutralized with *N* HCl (16.3 ml.). On standing, luteose (19 g.),  $[\alpha]_D^{20} -33^\circ$ , separated and a further sample (79 g.) was obtained by the addition of absolute alcohol up to 75%. The combined products were washed with alcohol and dried in a vacuum.

*Acetylation of luteose.* Luteose (75 g.) was suspended in a mixture of water (100 ml.) and pyridine (750 ml.), and to this acetic anhydride (750 ml.) was added cautiously. After being kept overnight at  $15^\circ$  the mixture was heated at  $90^\circ$  for 6 hr. The resultant gel-like solid was filtered off, washed thoroughly with pyridine and with water until free from acid and dried in a vacuum. The product was a yellow powder, acetyl content 23%. It was further acetylated with acetic anhydride and glacial acetic acid containing chlorine and sulphur dioxide and the product was isolated by pouring the reaction mixture into water. After washing with water and drying in a vacuum, acetyl luteose (70 g.) was obtained as a white powder  $[\alpha]_D^{20} -5^\circ$  in chloroform, average acetyl content, 45.4% and on fractionation it appeared to be essentially homogeneous.

*Methylation of acetyl luteose.* Acetyl luteose (60 g.) was methylated with dimethyl sulphate (290 ml.) and 40% KOH (630 ml.) in the presence of acetone in the usual manner.  $K_2SO_4$  was removed by filtration, the filtrate neutralized and evaporated to dryness in a vacuum. The residue was extracted with chloroform under reflux, the extract dried over anhydrous  $MgSO_4$ , and the solvent removed yielding a brown syrup (34.5 g.) OMe, 31.8%. This material was again methylated six times in a similar manner, the final product (33 g.) having OMe, 42.4%. Further methylation did not increase the methoxyl content. The methylated product (50 g.) was exhaustively extracted with acetone in a Soxhlet apparatus leaving an insoluble methylated luteose (44 g.) as a white powder,  $[\alpha]_D -32^\circ$ , OMe, 41.5%. The acetone extracts containing a soluble methylated polysaccharide were retained for later investigation.

*Hydrolysis of methylated luteose.* The compound (35 g.) was very stable to the usual hydrolytic agents but it was satisfactorily hydrolysed by fuming HCl at  $0^\circ$  during 156 hr., at the end of which time a final equilibrium value  $[\alpha]_D +18^\circ$  was reached. The HCl was removed by aeration followed by addition of  $BaCO_3$  in the presence of charcoal; after filtration the pale yellow filtrate and washings were combined and extracted with chloroform, the extracts dried over anhydrous  $MgSO_4$  and evaporated to a syrup, fraction A<sub>1</sub> (8.8 g.). The aqueous mother liquor was evaporated to dryness and the residue exhaustively extracted with chloroform in a Soxhlet apparatus. Removal of the chloroform by distillation

left a syrup, fraction B<sub>1</sub> (24.0 g.). Fractions A<sub>1</sub> and B<sub>1</sub> were separately converted into the glucosides by heating with 1% methyl-alcoholic HCl and these were fractionated in a high vacuum using a Widmer column. The following *main* fractions were finally obtained.

Fraction	Bath temp. 0.02 mm.	$n_D^{17}$	Yield g.	% OMe	Remarks
A <sub>1</sub> . Chloroform extract (8.8 g.)					
1	130–5°	1.4573	4.24	52.1	Crystallized
2	130–5	1.4581	1.14	52.1	Crystallized
3	Residue	—	3.58	—	Crystallized
B <sub>1</sub> . Water-soluble material (26 g.)					
4	135–40	1.4608	15.65	—	Crystallized
5	140–50	1.4733	2.49	—	Viscous syrup
6	150–60	1.4740	2.56	—	Viscous syrup

The crystalline material, m.p. 92–93°,  $[\alpha]_D - 21^\circ$  (c, 1.0) in water, was drained on a tile and recrystallized from light petroleum. It was identified as 2:3:4-trimethyl  $\beta$ -methylglucopyranoside. No depression of m.p. was given in admixture with an authentic specimen and it was oxidized by bromine water to 2:3:4-trimethyl  $\delta$ -gluconolactone,  $[\alpha]_D + 82^\circ \rightarrow +33^\circ$  in 26 hr. Despite careful search in the syrupy mother liquors no tetramethyl methylglucopyranoside could be detected. Fractions 5 and 6 were methylated further and hydrolysed to give tetramethyl glucose (80% yield), m.p. 92–93°.

*Luteose. Sample II (M. Stacey's preparation)*

The above investigation was repeated on another sample of luteose. The luteic acid used for this sample was prepared by Mr G. Smith, in the manner previously described, from cultures of a different strain of *P. luteum*, L.S.H.T.M. Cat. No. P 211, which grows more quickly. This strain was purchased from the Centraalbureau voor Schimmelcultures, Baarn, Holland, in March 1937. The metabolism solution was evaporated to a small bulk and the crude polysaccharide precipitated by addition of alcohol. This material had  $[\alpha]_D - 32^\circ$  (c, 1.1) ash, 5.5%. On fractionation of this substance (5.5 g.) from water by addition of alcohol the following samples were obtained.

Fraction	Vol. of alcohol added	Yield g.	$[\alpha]_D$ corr. for ash	% ash
1	—	0.2	-32°	0.4
(insol. in water)				
2	5	3.1	-34	1.2
3	8	1.0	-34	0.8
Mother liquor	—	0.4	-30	10.3

Luteic acid (5 g.) was warmed at 50° for 6 hr. with *N*/10 NaOH (100 ml.). The solution was cooled, neutralized with the exact equivalent of *N* HCl and poured into alcohol (6 vol.). The "luteose" which was precipitated was isolated (3.8 g.). It was partly soluble in cold water,  $[\alpha]_D^{20} - 32^\circ$ , and had ash content 10.0%. The alcohol mother liquors were evaporated to a syrup, acidified with H<sub>2</sub>SO<sub>4</sub> and extracted for 24 hr. with ether in a continuous extractor. Removal of the ether left a crystalline mass (1.0 g.) which was purified by sublimation and shown to be malonic acid (m.p. 133–134°). Extraction of the "luteose" (above) with cold water yielded a non-viscous soluble polysaccharide (0.6 g.),  $[\alpha]_D - 25^\circ$  (c, 1.2) which failed to give a precipitate with Fehling's solution in contradistinction to purified luteose which could be precipitated quantitatively as a copper luteose complex by Fehling's solution.

Luteose was prepared as follows: crude luteic acid (10 g.) was dissolved in water (200 ml.) and centrifuged, yielding an insoluble deposit (0.6 g.). The clear supernatant liquid was mixed with Fehling's solution (50 ml.) and after stirring for a few minutes a copious gelatinous precipitate separated. A further amount of Fehling's solution (100 ml.) together with distilled water (300 ml.) was added, the mixture thoroughly stirred, and kept for an hour. The precipitate was spun off, washed thoroughly with cold distilled water (all washings and supernatant liquids being retained), and dissolved in *N* acetic acid (100 ml.). Luteose was precipitated in the form of a granular powder by addition of 4 vol. of alcohol. It was separated and washed thoroughly with alcohol containing acetic acid, until completely free from copper salts, and it was finally washed with *N*/10 acetic acid and dried with absolute alcohol and ether (yield, 4.0 g.).

Luteose had the following properties: A white granular powder insoluble in cold water, soluble in sodium hydroxide,  $[\alpha]_D^{20} -38^\circ$  (c, 1.1): iodine number negligible, non-reducing to Fehling's solution: could not be separated into fractions having different properties. Crystalline glucose in 95% yield was obtained on hydrolysis with 2*N*  $\text{H}_2\text{SO}_4$  at  $95^\circ$ .

*Isolation of a second polysaccharide from P. luteum Zukal* (Cat. No. P. 211). The supernatant liquors from the Fehling's solution above were combined, carefully neutralized with 5*N* acetic acid and concentrated to a syrup in a vacuum. Addition of alcohol gave an impure polysaccharide in the form of a glutinous mass. It was purified by repeated precipitation from aqueous solution by alcohol and was finally obtained as a fine white hygroscopic powder, readily soluble in water,  $[\alpha]_D^{20} -25^\circ$  (c, 2.4), forming non-viscous solutions. On hydrolysis with 2*N*  $\text{H}_2\text{SO}_4$  at  $95^\circ$  for 2 hr. an equilibrium rotation,  $[\alpha]_D^{20} +56^\circ$  was reached while among the products of hydrolysis crystalline *d*-galactose (66% yield) and mannose (20% yield) identified and estimated as mannose phenylhydrazone were isolated. The polysaccharide (which we propose to name "galuteose") was probably a galactan of a type similar to varianose [Haworth *et al.* 1935]. Its methylation proceeded in a manner comparable with that of varianose and the methylated product was readily soluble in acetone. Further investigations on this material will be described later.

*Methylation of luteose.* Luteose (10 g.) was dissolved in NaOH solution and methylated (four times) with dimethyl sulphate in the presence of acetone. The partly methylated luteose (OMe, 38%) was obtained in the form of a white powder which was insoluble in all solvents except chloroform. It was precipitated in a finely divided condition from dilute chloroform solution by addition of light petroleum and was remethylated with dimethyl sulphate and 30% NaOH in the presence of dioxane. Twelve methylations gave a product (9.2 g.), OMe, 43.8%,  $[\alpha]_D^{20} -30^\circ$  in chloroform. Repeated attempts using different conditions were made to increase the methoxyl content but without success. Fractionation of methylated luteose (9.0 g.) was carried out as usual by precipitation from chloroform solution by light petroleum.

Fraction	Yield g.	$[\alpha]_D$ in chloroform	$\eta_{sp}$	% OMe	% ash
$F_1$	5.2	$-31^\circ$	0.123	43.8	0.12
$F_2$	2.2	$-29$	0.100	43.6	0.16
$F_3$	1.5	$-27$	0.108	43.6	0.18

These properties provide evidence of the essential homogeneity of methylated luteose.

*Hydrolysis of methylated luteose.* Methylated luteose (38 g.) was hydrolysed by fuming HCl at  $0^\circ$  by the method described previously. The methyl glucosides

were prepared and on distillation in a high vacuum the following principal fractions were isolated.

Fraction	B.P. (bath temp.) 0.02 mm.	$n_D^{20}$	Yield g.	% OMe	Remarks
A <sub>2</sub> . Chloroform extract (10.0 g.)					
1st drop	115°	1.4550	—	—	Crystallized
2nd drop	118	1.4560	—	—	"
I	120-2	1.4560	1.1	50.9	"
II	120-2	1.4560	4.1	50.8	"
III	122-3	1.4560	4.0	51.0	"
B <sub>2</sub> . Water-soluble material (26 g.)					
1st drop	122-3	1.4565	—	—	"
IV (main bulk)	122-6	1.4568	22.1	50.9	"
V	125-8	1.4569	0.5	—	"
VI	135-45	1.4700	3.2	41.7	—

*Identification of the trimethyl fractions.* Fraction I (0.5 g.) was hydrolysed to the sugar which was isolated as a syrup (0.45 g.), OMe, 41.4% (theory for trimethyl glucose 41.9%),  $n_D$  1.4710,  $[\alpha]_D + 60^\circ$  in water (c, 0.8). It was converted into the anilide [cf. Peat *et al.* 1938] which was obtained in quantitative yield, M.P. 145–146° alone or in admixture with an authentic specimen of 2:3:4-trimethyl glucopyranose anilide. No evidence of the presence of tetramethyl glucose anilide was obtained.

The crystalline fractions were combined, drained on a porous tile and recrystallized from ether-light petroleum, M.P. 93–94° alone or in admixture with a specimen of 2:3:4-trimethyl  $\beta$ -methylglucopyranoside. (Found: C, 50.8; H, 8.5; OMe, 52.1%.  $C_{10}H_{20}O_6$  requires C, 50.8; H, 8.5; OMe, 52.5%.) A sample was hydrolysed in the usual way to syrupy trimethyl glucose which, on heating with the equivalent of aniline in alcoholic solution, yielded 2:3:4-trimethyl glucopyranose anilide M.P. and mixed M.P. 145–146°.

The tile was extracted with chloroform which was distilled off leaving a syrup from a portion of which 2:3:4-trimethyl glucopyranose anilide was prepared as above. A second portion was hydrolysed and the product oxidized with bromine to 2:3:4-trimethyl  $\delta$ -gluconolactone,  $[\alpha]_D^{21} + 33^\circ$  (equilibrium value, c, 0.9). This lactone was treated with nitric acid (sp. gr. 1.26) at 100° for 1 hr. The excess acid was removed by distillation in steam and the oxidation products converted into the methyl esters by boiling with 1% methyl-alcoholic HCl. The main product (60% yield) distilled at 140–145°/0.01 mm., had  $n_D^{21}$  1.4485, crystallized on nucleation with 2:3:4-trimethyl saccharolactone methyl ester and had M.P. and mixed M.P. 106–107°.

*Examination of the dimethyl fractions.* Fraction VI showed  $[\alpha]_D + 91^\circ$  (c, 1.2 in water). A portion (0.5 g.) was methylated three times with silver oxide and methyl iodide giving a syrupy product, OMe, 61.0%,  $n_D^{22}$  1.4440, which was hydrolysed with HCl (6%). The crystalline hydrolysis product (0.4 g.) isolated in the usual way was 2:3:4:6-tetramethyl glucopyranose, M.P. 84° alone or in admixture with an authentic specimen. A further portion (1.0 g.) was hydrolysed with HCl (6%),  $[\alpha]_D^{21} + 95^\circ$  changing to  $[\alpha]_D^{22} + 70^\circ$  (equilibrium value) in 6 hr. The syrupy product (0.6 g.) had  $[\alpha]_D + 72^\circ$  in water, OMe, 28.4% (dimethyl glucose requires OMe, 33.6%). Under the usual conditions this product yielded an anilide in 20% yield. This had M.P. 120–130°, and was apparently a mixture. Its separation by fractional crystallization was not satisfactorily accomplished and the exact configuration of the dimethyl fraction is under investigation.

*Molecular weight of luteose*

Osmotic pressure measurements to determine the molecular weight were carried out by Mr W. T. Chambers to whom we are greatly indebted. A value corresponding to 84 glucose units was obtained.

## SUMMARY

Luteose, the neutral polysaccharide produced by elimination of the malonyl residues from luteic acid, a metabolic product of *Penicillium luteum* Zukal, has been shown to be constituted mainly of  $\beta$ -glucose units linked through the 1:6-positions. The molecule may be a terminated linear chain but determination of its molecular weight (84 units) by osmotic pressure measurements and the presence of dimethyl glucose (10 %) among the products of hydrolysis of methylated luteose, indicates that the molecule is more likely to be of the closed chain type.

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## XXXIV. ORGANIC ANIONS OF HUMAN ERYTHROCYTES

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IN their efforts to simplify the two phase physico-chemical system of the blood, Henderson [1928] and other authors [Van Slyke *et al.* 1923; Dill *et al.* 1937] assumed that anions in normal human erythrocytes other than  $\text{Cl}^-$ ,  $\text{HCO}_3^-$  and  $\text{Hb}^-$  (designated by Henderson  $\text{X}^-$ ) were usually insignificant in amount. This assumption was supported by analyses which seemed to show that any excess of base over  $\text{Cl}^- + \text{HCO}_3^- + \text{Hb}^-$  was negligible. It is now clear, however, that the values assigned to  $\text{Hb}^-$  were too high either because it was assumed that the buffering powers of human and horse haemoglobins were similar or because the data were derived from haemolysed erythrocyte solutions instead of from solutions of pure Hb. Maizels & Paterson applied Adair's [1925] titration curves to the data of Henderson [1928] and of Dill *et al.* [1937], and found that the base was actually greater than  $\text{Cl}^- + \text{HCO}_3^- + \text{Hb}^-$ ; they confirmed these observations [1937] experimentally by analysing a series of erythrocyte solutions and calculating  $\text{Hb}^-$  from their own electrometric titrations of Hb.

It thus became necessary to assume the presence of another anion which, following Henderson, they called  $\text{X}^-$ , and which further appeared to be roughly inversely proportional to the Hb content per litre of original cells, being much greater in the haemoglobin-deficient cells of certain anaemias than in normal erythrocytes. In the latter,  $\text{X}^-$  was about equal to  $\text{Hb}^-$ , while in anaemic cells it might be twice as great as  $\text{Hb}^-$ . Further, knowing the Hb content per 1000 ml. cells it was found possible to foretell the probable value of  $\text{X}^-$  to within  $\pm 15\%$  (Fig. 1).

It will be realized that the amount of  $\text{X}^-$  depends ultimately on the titration curve of  $\text{Hb}^-$  and although the figures found by Adair [1925] and by Maizels & Paterson [1937] are probably correct, in order to remove any ambiguity on this score an effort was made to demonstrate the presence of  $\text{X}^-$  in the absence of Hb by analysing the dialysate of laked cell solutions. A number of experiments were done but as the fuller and more satisfactory experiments carried out subsequently are described later in this paper, the results of the original experiments will only be summarized here.

The dialysate of laked cell solutions contained a marked excess of base over  $\text{Cl}^-$  and  $\text{HCO}_3^-$ , so that the presence of  $\text{X}^-$ , apparently responsible for 30% of total base-binding, in dialysates was confirmed. The titration curve of the dialysate showed marked buffering between pH 6 and 7 and in this resembled the titration curve of phosphate and the phosphoric esters [Meyerhof & Suranyi, 1926] but while the buffering power of phosphate greatly decreases at  $\text{pH} > 7$ , the buffering power of the dialysate was maintained until well beyond pH 9 and this part of the curve recalled the titration curve of glutathione [Pirie & Pinhey, 1929]. Analysis of the dialysates showed in fact that organic phosphorus and SH— groups were present. It therefore seemed reasonable to assume that phosphoric esters and glutathione might account in part at least for  $\text{X}^-$ . Support for this view was forthcoming from results already available in the literature

which may be summarized as follows: human erythrocytes contain about 70 mg./100 ml. glutathione [Platt, 1931] which would bind 1.1 equiv. base per mol. at the pH of the erythrocyte (7.5 at 20°), and about 50 mg./100 ml. acid-soluble P [Kay *et al.* 1927; Kerr & Daoud, 1935; Rapoport, 1937] which binds 2.4 equiv. base per atom P at pH 7.5 [Farmer & Maizels, 1938]: approximately 25% is easily hydrolysable phosphate, probably pyrophosphate corresponding in whole or in part to adenosine triphosphate [Kerr & Daoud, 1935], which binds 1.3 equiv. base per atom P, while the residue consists of inorganic phosphate, hexosephosphate and other phosphates binding little < 2 equiv. base per atom P at pH 7.5. At a conservative estimate then cell P should bind 1.9 equiv. base per atom P so that theoretically cell P and glutathione should bind together 32 m. equiv. base per 1000 ml. cells.

Since this is about twice as great as the amount of base bound by Hb, it is extremely remarkable that workers on the acid-base equilibrium of the blood should hardly have taken the base-binding properties of phosphoric esters into account at all. Comments have indeed been made on the compensatory decrease in cell P which occurs in experimental acidosis [Haldane *et al.* 1924; Kay, 1924; Rapoport, 1937], but the only direct reference we have found is by Peters & Van Slyke [1931] who, in contrast with Van Slyke's statements of 1923, were able to say in 1931: "it has generally been assumed that proteins are the chief

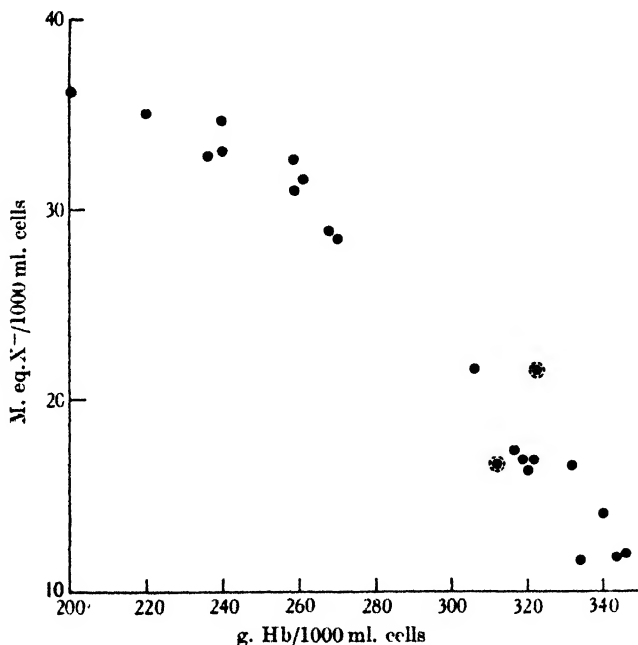


Fig. 1.

buffers in cells; but it is quite possible that organic phosphates are more important". We shall see later that this suggestion is incorrect since, the importance of the organic phosphates lies primarily in their base-binding and not in their buffering powers. In view of the amount of base that could theoretically be bound by the acid-soluble P of cells, we have ourselves carried out a number of new analyses on normal and anaemic cells and have compared

the theoretical amount of base bound by cell phosphate and glutathione with the amount probably bound by  $X^-$ , the non-haemoglobin organic anions of erythrocytes, as determined from Fig. 1.

### Methods

Erythrocytes were separated for analysis by centrifuging blood which had been treated with phosphate-free heparin (Hoffmann-la Roche). Whole blood was kept on ice as far as possible, and despite the delay in adding trichloroacetic acid Table I shows that little hydrolysis of phosphoric esters occurred. Inorganic P and total acid-soluble P were determined according to Martland & Robison [1926]. Easily hydrolysable P was estimated by boiling the trichloroacetic acid filtrate for 7 min. with  $N H_2SO_4$ , while the glutathione was estimated iodimetrically in acid solution [Platt, 1931]. The results are summarized in Table I and are expressed in mg. or m. equiv. per 1000 ml. cells.

Table I

Case	Hb g. blood	Hb g. cells	Total acid-sol. P mg.	Hydro- lysable P mg.	Inor- ganic P mg.	Gluta- thione (G1) mg.	P + G1 m. equiv.	$X^-$ m. equiv.
1. Normal	156	335	485	93	17.4	680	32	14
2. Normal	148	333	465	125	12.1	810	31.5	16
3. Hyperpiesis	118	325	550	94	30.5	790	36.5	17
4. Normal	124	320	527	124	21.4	600	34	18
5. Normal	152	318	510	—	20.7	740	34	19
6. Hyperpiesis	114	312	510	—	20.1	730	33.5	21
7. Normal	150	312	540	115	18.8	980	36.5	21
8. Normal	146	308	462	103	16.9	800	31	21
9. Normal	136	302	510	122	23.0	840	34	23
10. Prostatectomy	141	301	457	100	17.0	970	31.5	23
11. Microcytic anaemia	88	280	680	115	22.5	1040	45.5	28
12. Microcytic anaemia	59	260	592	114	18.1	910	39.5	31
13. Microcytic anaemia	70	259	691	128	22.6	1050	46	31
14. Microcytic anaemia	70	230	702	130	22.3	1140	47	35
15. Microcytic anaemia	52	229	675	—	17.0	1130	45.5	35
16. Microcytic anaemia	36	203	625	118	16.1	1350	43	38
17a. Macrocytic anaemia	50	283	610	110	15.2	1130	41.5	?
17b. Macrocytic anaemia	63	267	692	100	19.2	1220	46.5	?
18. Acholuric jaundice	134	343	450	106	21.5	600	29.5	10

The cases fall into three groups: 10 without anaemia, 6 with hypochromic microcytic anaemia and 1 with macrocytic anaemia. The two main groups are placed in order of their Hb content per 1000 ml. cells. The analyses of normal cells correspond with those of other workers; in microcytic anaemia both acid-soluble P and glutathione are increased but the correlation between these increases and Hb either in unit volume of cells or whole blood is not close. The possibility of increases in P and glutathione being associated with reticulocytosis was considered but since reticulocytosis in the first 16 cases never exceeded 2% this could not have been a cause. Again, in case 17, 0.25% of reticulocytes were present at the first examination (a) and 25% at the second (b), but glutathione was only increased by 10%. Finally, the last case in the series had 4.8% reticulocytes but total P and glutathione were the lowest recorded. At present the relation of high glutathione values to anaemia therefore remains obscure. The function of the increased phosphoric esters is a little more clear; in microcytic anaemia Hb and base bound by Hb decrease while cell water increases; phosphoric esters will help to maintain base-binding and, being osmotically active, will support the osmotic pressure of the cell contents. On the other hand, phosphates are not good buffers at  $pH > 7$  and they probably play little part in

cell buffering in normal or anaemic cells: assuming that 1000 ml. normal cells contain 320 g. Hb binding 20 m. equiv. base at pH 7.25, and that there are also present 500 mg. acid-soluble P binding 30 m. equiv. base at pH 7.25, then it would require 4 m. equiv. base to shift a solution of the Hb alone to pH 7.35 and only 0.6 m. equiv. to effect a corresponding change in the phosphoric esters alone. In anaemia on the other hand the buffering power of the phosphoric esters is a little more significant.

If cell phosphate bound its full theoretical amount of base at cell pH it would combine with nearly twice as much base as Hb itself. Moreover, since plasma contains very little phosphoric ester, the acid-soluble phosphates within the erythrocyte should act as non-permeating anions whose effect on the distribution of permeating ions should far outweigh that of haemoglobin. For this reason equations dealing with the distribution of water and electrolytes between cells and plasma, which refer solely to Hb as the major non-permeating ion are likely to have no more than comparative significance.

#### *Base bound by $X^-$ and the phosphoric esters*

If the phosphoric esters and glutathione in the cells were to bind their full theoretical quota of base, then  $X^-$ , that is  $B^+ - Cl^- - HCO_3^- - Hb^-$ , should be equal to or greater than the equivalence of the phosphoric esters + glutathione. Actually, comparison of the last two columns in Table I shows that the reverse is the case and this can only arise in one of four ways: (a) less than 1.9 equiv. base is bound per atom P; (b) an unidentified excess of base is present in the erythrocytes; (c) the value of  $B^+ - Cl^- - HCO_3^- - Hb^-$  is too small owing to some analytical error; (d) phosphoric esters and/or chloride exist in some form in which they fail to bind base. Consideration of these possibilities leads to the following conclusions. (a) It is probable that P does in fact bind at least 1.9 m. equiv. base per atom at cell pH. This was confirmed in the following way: a trichloroacetic acid filtrate of erythrocytes was neutralized with ammonia using methyl orange as an indicator. Basic lead acetate and alcohol were added to precipitate the phosphates. The deposit was well washed, first with dilute ammonia solution to remove acetate, trichloroacetate, Na and K, and then with alcohol and water to remove ammonia. Lead was removed with  $H_2S$  and the filtrate evaporated *in vacuo* at 40°. The residue was taken up in water and found to be free from ammonia and sulphur. The P content of this solution was estimated and it was then titrated electrometrically at 38°. It was found that each atom of P corresponded to 1.86 equiv. base at pH 7 and 2.03 equiv. at pH 7.5. (b) There is no evidence of the presence of any unidentified excess of base, but if such were present, then it should be recovered by electrodialysis. (c) The values for base and chloride recorded by Maizels & Paterson [1937] were obtained by well-tried methods which gave consistent results agreeing with the figures of other workers. Of these, the data of Dill *et al.* [1937] may be quoted. These workers, using entirely different methods, give the following average figures of twelve individuals: base, 11.0; Cl, 5.6;  $HCO_3^-$ , 1.75 m. equiv. per 100 ml., whence  $X^- + Hb^- = 3.6$  m. equiv./100 ml.

If  $Hb^-$  is calculated according to the data of Dill *et al.*,  $X^- = -0.07$  m. equiv., but employing the more accurate data of Adair [1925] or of Maizels & Paterson [1937] for the buffering power of reduced Hb, then  $Hb^-$  may be calculated as follows:

$$Hb^- = Hb \times (pH - I.P.) \times B_r$$

where Hb equals the amounts of haemoglobin present in g.; pH its pH; I.P. the isoelectric point of reduced Hb (6.81 at 38°) and  $B_r$  the buffering power of

reduced Hb per g., which is 0.138 m. equiv. per pH unit between 6.8 and 7.3. Then in the experiments of Dill *et al.*,  $\text{Hb}^- = 33.5 \times (7.167 - 6.81) \times 0.138 = 1.7$  m. equiv. per 100 ml. cells, and  $\text{X}^- = 3.6 - 1.7 = 1.9$  m. equiv. per 100 ml. blood. This agrees well with the corresponding figure given by Maizels & Paterson, and there is therefore no reason to ascribe the apparent excess of base to analytical error. (d) There remains the fourth possibility that phosphoric esters and/or chloride within the cell bind less base than they do in simple aqueous solution. This might possibly occur through some form of complex association and at present represents the most likely explanation; experimental evidence on the point is however so far lacking. One further point in this connexion does indeed emerge from Table I: although  $\text{X}^-$  and acid-soluble phosphate are both increased in hypochromic microcytic anaemia, the increase in  $\text{X}^-$  is relatively the greater and this suggests the possibility that the increase in  $\text{X}^-$  may be accomplished by the diversion of phosphate from a complex to a simple form.

In the attempt to obtain direct evidence as to the state of phosphoric esters within the cell, laked cell solutions were dialysed with the object of determining whether  $\text{X}^-$  and the phosphoric esters dialysed at the same rate and whether the apparent excess of total phosphate over  $\text{X}^-$  was to be observed in the dialysate as well as in the dialysed solutions.

*$\text{X}^-$  and phosphoric esters in dialysed cell solution and dialysate*

Human erythrocytes were washed 5 times with isotonic KCl solution, packed by centrifuging and the supernatant KCl removed with a capillary pipette. The cells were then mixed with half their volume of distilled water, vigorously shaken with a similar volume of ether and recentrifuged. A compact ethereal scum rose to the surface, containing lipins, cell stromata and other substances. The subjacent clear laked cell solution was removed and freed from ether by evaporation *in vacuo* at room temperature; it was then dialysed in cellophane bags against one-third its volume of water, the systems being mixed by inversion every 2 hr. Dialysis was carried out in two cases for 18 hr. and in one case for a week. In no case was true equilibrium attained. Finally, the dialysed cell solution and dialysate were analysed. Details of the methods employed are given in the appendix together with a full statement of all the data obtained in Exp. 2. The results of all three experiments are summarized in Table II, where the following are set out:

(a) No. of hr. of dialysis; (b) vol. of cell solution and of dialysate; (c) Hb in g. per 1000 ml.; (d) electrometric measurement of pH; (e)  $\text{Hb}^-$  in m. equiv. per 1000 ml.  $\text{Hb} \times (\text{pH} - \text{i.p.}) \times \text{B}_r$ ; (f) Cl in m. equiv. per 1000 ml.; (g)  $\text{HCO}_3^-$  in m. equiv. per 1000 ml.; (h) base bound by glutathione ( $\text{Gl}^-$ ) per 1000 ml. at the experimental pH (calculated from the titration curves of Pirie & Pinhey [1929]); (i) total base (this is one of the most crucial as well as the most difficult estimations; it was carried out by three different methods: (i) electrolytically; (ii) as the sum of  $\text{K} + \text{Na} + \text{Mg} + \text{NH}_4$ ; (iii) as sulphate by the benzidine precipitation method; (j)  $\text{X}^-$  in m. equiv. per 1000 ml. (in this case,

$$\text{X}^- = \text{Base}^+ - \text{Cl}^- - \text{HCO}_3^- - \text{Hb}^- - \text{Gl};$$

$\text{CO}_2$  and glutathione are quite small and may well be neglected; they are, however, included for the sake of completeness); (k) inorganic phosphate; (l) total acid-soluble P mg. per 1000 ml.; (m) total acid-soluble phosphate in m. equiv. per 1000 ml.: it has been assumed that 1 atom acid-soluble P binds 1.9 m. equiv. base at pH 7.2; if all cell phosphate is fully ionized then base bound by  $\text{X}^-$  should be as great as that bound by total phosphate or greater.

Table II.  $T. = 38^{\circ}$ 

No. of hr. dialysis	Exp. 1		Exp. 2		Exp. 3	
	Cell soln.	Dialysate	Cell soln.	Dialysate	Cell soln.	Dialysate
	18	18	18	18	168	168
Vol. ml.	50.4	34	53	38	63.5	33
Hb colorimetric g./1000 ml.	11.4	0	17.7	0	13.4	0
Hb gasometric g./1000 ml.	—	—	18.1	—	—	—
pH	7.29	7.68	7.19	7.65	7.19	7.57
Hb <sup>+</sup> m. equiv./1000 ml.	7.5	0	9.7	0	7.0	0
Cl <sup>-</sup> m. equiv./1000 ml.	21.2 $\pm$ 0.5	25.8 $\pm$ 0.4	33.1 $\pm$ 0.2	40.2 $\pm$ 0.2	32.3 $\pm$ 0.5	37.5 $\pm$ 0.5
HCO <sub>3</sub> <sup>-</sup> m. equiv./1000 ml.	0.2	0.14	0.2	0.1	0.1	0.1
Gl <sup>-</sup> m. equiv./1000 ml.	0.64	0.85	0.58	0.66	0.57	0.67
Total base:						
Electrolytic m. equiv./1000 ml.	32.1 $\pm$ 0.4	31.9 $\pm$ 0.2	48.4 $\pm$ 0.6	47.5 $\pm$ 0.5	48.1 $\pm$ 0.5	52. $\pm$ 1.0
K + Na + Mg + NH <sub>4</sub> m. equiv./1000 ml.	—	—	48.0 $\pm$ 1.0	47.6 $\pm$ 0.2	—	—
as SO <sub>4</sub> m. equiv./1000 ml.	—	—	48.4 $\pm$ 1.2	—	—	—
X <sup>-</sup> m. equiv./1000 ml.	2.6 $\pm$ 1.0	5.1 $\pm$ 0.6	4.7 $\pm$ 1.2	6.4 $\pm$ 0.7	8.1 $\pm$ 1.0	13.8 $\pm$ 1.5
P inorganic mg./1000 ml.	24.7	12.2	15.0	6.9	59.5	54.3
P acid-sol. mg./1000 ml.	144.3 $\pm$ 1.3	70.1 $\pm$ 0.9	224 $\pm$ 1.0	79.9 $\pm$ 0.9	185 $\pm$ 3.0	140 $\pm$ 1.5
Total acid-sol. phosphate	8.8 $\pm$ 0.1	4.3 $\pm$ 0.06	13.7 $\pm$ 0.06	4.9 $\pm$ 0.06	11.3 $\pm$ 0.2	8.6 $\pm$ 0.1
- - RHPO <sub>4</sub> <sup>-</sup> m. equiv./1000 ml.						
X <sup>-</sup> /—RHPO <sub>4</sub> <sup>-</sup>	0.3	1.2	0.34	1.3	0.7	1.6

The following conclusions may be drawn from Table II.

(1) The dialysate is much more alkaline than the dialysed cell solution; this is to be expected since the cell solution contains the non-penetrating anions Hb and organic phosphates.

(2) Chloride, glutathione, base and X<sup>-</sup> diffuse rapidly.

(3) Acid-soluble phosphate diffuses slowly through cellophane; at 18 hr. the dialysate contains less than one-half the amount of acid-soluble P present in the cell solution, while after 7 days' dialysis, P in the dialysate is only two-thirds of that in the cell solution. It will also be noted that the ratio of inorganic to total acid-soluble P rises as the period of dialysis increases and this is doubtless because hydrolysis by cell phosphatases is prolonged.

(4) When all allowance is made for experimental errors, it is still true that after 18 hr. dialysis, the theoretical amount of base that could be bound by total phosphate in the cell solution is twice as great as that bound by X<sup>-</sup>, while in the dialysate it is slightly less. The observations gain strength from the fact that the same methods, often in quadruplicate, were used on both solutions which differed only in that one contained haemoglobin while the other did not. Further, of all the values given in Table II, there is only one assumed figure and that is the pH at which reduced haemoglobin is isoelectric. This is usually taken to be 6.81; if in fact the isoelectric point were lower, then the ratio of X<sup>-</sup> to phosphate would be even less; if on the other hand the isoelectric point were higher, then X<sup>-</sup> and phosphate would tend to become more nearly equal, though X<sup>-</sup> would still be much less than phosphate unless the isoelectric point of haemoglobin at 38° were at pH > 7. Rejecting the latter remote possibility, one is forced to conclude that at least half of the phosphate in cells is not binding base but is complexly combined. The only possible alternative to this view is the unlikely one that chloride and not phosphate is complexly combined.

Although the systems are not in equilibrium, it is still of interest to compare the concentrations of ions in the cell solution and in the dialysate. Concentrations are obtained by dividing the known ion contents by the amount of water present

in the corresponding phase. In the case of cell solutions a figure has been subtracted from the measured total content of water equal to 0.2 ml. bound water per g. Hb (Table III).

Table III

	Exp. 1	Exp. 2	Exp. 3
$[\text{H}_2\text{O}]$ cell soln./ $[\text{H}_2\text{O}]$ dialysate	0.85	0.86	0.85
$[\text{H}^+]$ cell soln./ $[\text{H}^+]$ dialysate	2.45	2.82	2.40
$[\text{Base}]$ cell soln./ $[\text{Base}]$ dialysate	1.19	1.18	1.09
$[\text{Cl}^-]$ dialysate/ $[\text{Cl}^-]$ cell soln.	1.04	1.05	1.00
$[\text{Gl}]$ dialysate/ $[\text{Gl}]$ cell soln.	1.12	0.98	1.00
$\sqrt{[\text{Acid-sol. P}]} \text{ dialysate} / \sqrt{[\text{Acid-sol. P}]} \text{ cell soln.}$	0.64	0.56	0.80
$[\text{X}^-]$ dialysate/ $[\text{X}^-]$ cell soln. and $\sqrt{[\text{X}^-]} \text{ dialysate} / \sqrt{[\text{X}^-]} \text{ cell soln.}$	1.7 (1.3)	1.2 (1.1)	1.4 (1.2)

The phosphate ratio has been calculated on the assumption that P is divalent and the  $\text{X}^-$  ratio is shown both for a univalent and for a divalent anion.

It will be seen that the dialysate is much more alkaline than the cell solution and this has already been attributed to the excess of absolutely or relatively non-permeating anions, Hb and phosphate in the cell solution. But while  $[\text{H}^+]$  ratios are similar in all three experiments, the phosphate ratios are very different, for in Exps. 1 and 2, internal phosphate is twice external phosphate, while in Exp. 3, after a whole week's dialysis total acid-soluble phosphates within and without the cellophane bag are much more nearly equal. Hence changes in phosphate distribution are not accompanied by changes in the distribution of  $\text{H}^+$ , which suggests that phosphate in whole or in part is not acting as a relatively non-permeating anion and is in fact not acting as an anion at all. For, were phosphate in the cell solution acting as an anion a decrease in the phosphate ratio would bring about a decrease in the pH difference between cell solution and dialysate.

It will further be noted that  $[\text{Cl}^-]$  is greater in the dialysate and  $[\text{B}^+]$  greater in the cell solution; this is doubtless brought about by the excess of  $\text{Hb}^-$  in the cell solution. While  $[\text{B}^+]$  cell solution/ $[\text{B}^+]$  dialysate and  $[\text{Cl}^-]$  dialysate/ $[\text{Cl}^-]$  cell solution are in rough conformity, neither ratio agrees at all with the  $[\text{H}^+]$  ratio. Apart from the fact that all the systems are still far from equilibrium, there seems no explanation for this discrepancy. Phosphate has of course diffused very slowly and even after a week's dialysis is still greater in the cell solution.

Finally, we may note that while the dialysate/cell solution ratios of Cl, glutathione and  $\text{X}^-$  on the one hand, and the cell solution/dialysate ratio of base on the other, are in rough agreement, the phosphate ratios show no correspondence at all. Since the correspondence of  $[\text{Cl}^-]$  ratios is based on the assumption that  $\text{Cl}^-$  in cell solution is fully ionized, it is reasonable to believe that this assumption is correct; the phosphate ratios alone disagree with the other ion concentration ratios, while if one assumes that chloride and not phosphate in the laked cell solution is complexly combined, then both the phosphate and chloride ratios would be out of keeping with the ratio of base concentration.

#### SUMMARY

In normal human erythrocytes,  $\text{Base}^+$  is equal to the sum of  $\text{Cl}^- + \text{HCO}_3^- + \text{Hb}^-$  together with certain other complex organic anions symbolized by  $\text{X}^-$ .  $\text{X}^-$  binds about as much base as  $\text{Hb}^-$ , i.e. about 20 m. equiv. per 1000 ml. cells. Since erythrocytes contain phosphate (organic and inorganic) and glutathione, it is reasonable to assume that these substances may account for at least a part of  $\text{X}^-$ . Cell phosphate consists of diphosphoglycerate (65 %), adenosine triphosphate

(25 %) and other phosphates. These compounds in aqueous solution at pH 7.5 bind at least 1.9 m. equiv. base per atom of P. Since erythrocytes contain 500 mg. acid-soluble P per 1000 ml., this quantity of phosphate should bind 30 m. equiv. base, a quantity far in excess of  $X^-$ . It would therefore appear that much of the acid-soluble phosphate in cells is not ionized but is complexly combined.

If erythrocytes are dialysed,  $X^-$  is slightly greater than acid-soluble phosphate in the dialysate, but is very much less than acid-soluble phosphate in the dialysed cell solution. This shows that acid-soluble phosphate is fully ionized in the dialysate, but is only partially ionized in the laked cell solution.

In the erythrocyte of microcytic hypochromic anaemia, haemoglobin is decreased while  $X^-$ , glutathione, acid-soluble phosphate and water are all raised; this helps to maintain the base-binding and osmotic pressure of the anaemic cell. The function of cell phosphate as a buffer at the pH of the normal cell is relatively insignificant; in the anaemic cell at the same pH, it may be slightly more important.

## APPENDIX

### Methods

*Haemoglobin.* (a) Colorimetric, by Haldane's haemoglobinometer calibrated against a gasometrically standardized solution of blood. Duplicates should agree within  $\pm 2\%$ .

(b) Manometric [Peters & Van Slyke, 1931].

pH. This was measured in the hydrogen electrode after the method of Harington & Neuberger [1936]; the standardizing solution was 0.01 *N* HCl in 0.09 *N* NaCl and the values were sometimes confirmed with an acetate buffer. With these standard solutions reproducible E.M.F. were always obtained.

*Chloride* [Claudius, 1924]. Wet ashing is accomplished by boiling with  $HNO_3$  in the presence of  $KMnO_4$  as an oxidizing agent. The results obtained by this method have been compared with those obtained when  $H_2O_2$  is substituted as an oxidizing agent and also with the results obtained on a protein-free filtrate of cell solution treated with sodium tungstate and sulphuric acid, where chloride is estimated without a preliminary wet ashing. The agreement between all three methods was very close.

*Glutathione* [Platt, 1931]. Glutathione is estimated by iodimetric titration in acid solution. The material is first reduced with zinc and hydrochloric acid.

$CO_2$ -manometric.

*Total base* [Adair & Keys, 1934]. (a) At Dr Adair's suggestion we have used cellophane instead of collodion to close the cathode tube, and have found it best to electro-dialyse for a first period of 24 hr., and titrate. We have found it advantageous to replace the titrated sulphuric acid by fresh acid and then electro-dialyse for a further 24 hr., after which the second lot of acid is titrated. The first electro-dialysis recovers about 95 % of the base, and the second, the rest.

(b) Total base as  $K + Na + Mg + NH_4$ : Potassium was estimated by the method of Kramer & Tisdall [1921]; sodium, after Salit [1932]; magnesium by the technique described by Briggs [1924]. The test adopted for ammonia was nesslerization of a tungstate-sulphuric filtrate: it was negative in all cell solutions.

(c) Total base as sulphate, both directly [Fiske, 1922] and indirectly [Stadie & Ross, 1925].

Phosphorus was measured by the methods described by Martland & Robison [1926].

### Protocol of Exp. 2

*Hb.* Colorimetric. 176 and 178 g. per 1000 ml.

Gasometric. 181 g. per 1000 ml.

pH. Standard solution: 0.01 *N* HCl in 0.09 *N* NaCl.

E.M.F. standard HCl + calomel half cell = 363 mV. at 38°.

E.M.F. HCl =  $2.08 \times 61.7 = 128$  mV.

E.M.F. calomel half cell = 235 mV.

E.M.F. cell solution = (a) 678.5; (b) 678.5 mV. pH = 7.19.

E.M.F. dialysate = 707 mV. pH = 7.65.



*Chloride.* Solutions used: 0.5 ml.  $N/40$   $\text{AgNO}_3$ . 2 ml. of 2 in 15 dilution of cell solution or dialysate.

$\text{AgNO}_3$  = 1.200; 1.200; 1.205; 1.200; 1.200 ml.  $N/96.2$  KCNS.

$\text{AgNO}_3$  + cell solution = 0.35; 0.35; 0.36; 0.36; 0.36; 0.36 ml.  $N/96.2$  KCNS.

$\text{AgNO}_3$  + dialysate = 0.17; 0.16; 0.17; 0.16; 0.165; 0.16 ml.  $N/96.2$  KCNS.

Chloride m. equiv. per 1000 ml. cell solution =  $33.1 \pm 0.2$ .

Chloride m. equiv. per 1000 ml. dialysate =  $40.2 \pm 0.2$ .

$\text{HCO}_3$ . Cell solution:  $\text{CO}_2$  = 0.4 vols.  $\%$  = 0.2 m. equiv. per 1000 ml.

Dialysate:  $\text{CO}_2$  = 0.18 vols.  $\%$  = 0.1 m. equiv. per 1000 ml.

*Glutathione.* Cell solution: 2 ml. of 1 in 2 dilution in trichloroacetic acid

= 0.58 ml.  $N/1100$   $\text{I}_2$  = 0.53 ml.  $N/1000$   $\text{I}_2$ .

1000 ml. cell solution contain: 0.53 m. mol. = 0.58 m. equiv. glutathione at pH 7.2.

Dialysate. 1 ml. = 0.61 ml.  $N/1100$   $\text{I}_2$  = 0.55 ml.  $N/1000$   $\text{I}_2$

= 0.55 m. mol. = 0.66 m. equiv. glutathione at pH 7.65.

*Total base electrolytic.* Solutions: 1 ml.  $N/100$   $\text{H}_2\text{SO}_4$ . 1 ml. of 2 in 15 dilution of cell solution or dialysate.

1 ml.  $N/100$   $\text{H}_2\text{SO}_4$  = 1.000; 1.002; 1.004; 1.002 ml.  $N/100$  NaOH.

$\text{H}_2\text{SO}_4$  + cell solution = 0.365; 0.359; 0.351; 0.350 ml.  $N/100$  NaOH.

$\text{H}_2\text{SO}_4$  + dialysate = 0.366; 0.374; 0.364 ml.  $N/100$  NaOH.

Base m. equiv./1000 ml. cell solution =  $48.4 \pm 0.6$ .

Base m. equiv./1000 ml. cell solution =  $47.5 \pm 0.5$ .

*Total base as K + Na + Mg +  $\text{NH}_4$ .* K m. equiv./1000 ml. cell solution

= 41.2; 41.0; 41.2; 41.0; 41.0

=  $41.1 \pm 0.1$ .

K m. equiv./1000 ml. dialysate = 43; 43.1; 43; 43. =  $43 \pm 0$ .

To these must be added  $\pm 0.2$  corresponding to variations in the standard solutions

K m. equiv./1000 ml. cell solution =  $41.1 \pm 0.3$ .

K m. equiv./1000 ml. cell solution =  $43.0 \pm 0.2$ .

*Sodium.* Na m. equiv./1000 ml. cell solution = 3.7; 4.4. =  $4.05 \pm 0.3$ .

Na m. equiv./1000 ml. dialysate = 3.2; 3.2. =  $3.2 \pm 0$ .

*Magnesium.* Mg. m. equiv./1000 ml. cell solution = 3.1; 2.5 =  $2.8 \pm 0.3$ .

Mg. m. equiv./1000 ml. dialysate = 1.4.

*Ammonium.* Absent in cell solution and dialysate.

*Total base as sulphate.*

Direct: Cell solution m. equiv./1000 ml. = 47.2; 48.3; 48.7; 47.9.

Indirect: Cell solution m. equiv./1000 ml. = 49.4; 47.5; 49.6; 48.4.

Total base as sulphate in cell solution

m. equiv./1000 ml. =  $48.4 \pm 1.2$ .

*Total acid soluble phosphorus.* Three equal standards agreed exactly.

Cell solution mg./1000 ml. = 225; 225; 224; 225; 223.

Dialysate mg./1000 ml. = 80; 80.8; 79; 80.2.

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# XXXV. CATALASE ACTIVITY DURING THE DEVELOPMENT OF THE CHICK EMBRYO

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THE present note describes the changes in the catalase content of developing chicken embryos from the 4th to the 15th day inclusive.

The study of changes in enzyme content during development of embryo is of much interest in regard to the differentiation of metabolism. Edlbacher [1934] for instance observed a much greater concentration of arginase in young animals than in adult ones. The activity of the arginase of a chick embryo at different stages of development falls steeply from the beginning of development to the end. Edlbacher's suggestion is that arginase is concerned with a formation of arginine for the nucleoprotein of the nuclei.

## *Experimental methods*

The chick embryo was extracted from the egg, soaked for 1 min. in distilled water and accurately weighed. The embryo was then ground with powdered sand and the product dissolved in sufficient water, to make the final strength of

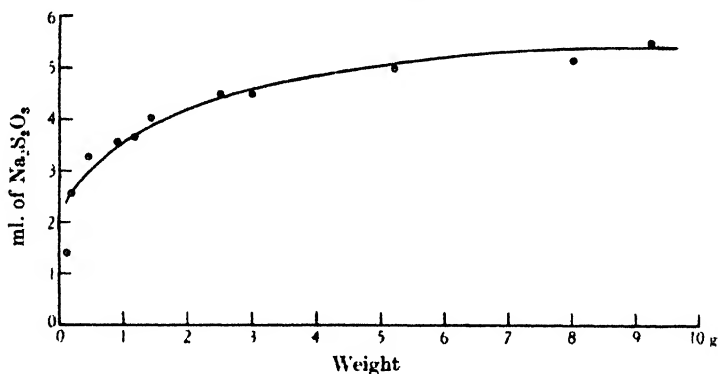


Fig. 1.

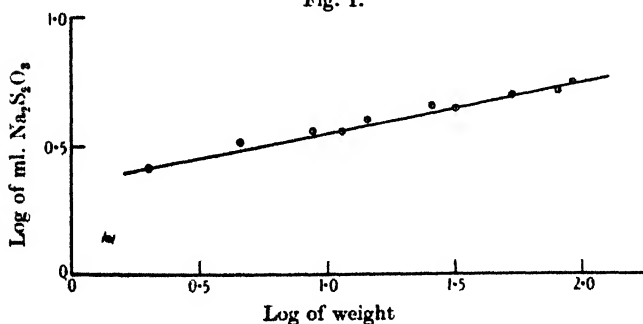


Fig. 2.

extract the same in all cases, viz. 10 mg. embryonic tissue per 1 ml. solution. The extracts were now centrifuged and 10 ml. of the fluid brought into contact with 20 ml. of 0.1 %  $\text{H}_2\text{O}_2$ . After 15 min., 5 ml. of the above were added to 5 ml. of 1 % KI and 3 ml. of 33 %  $\text{H}_2\text{SO}_4$ . The liberated  $\text{I}_2$  was determined by titration with  $N/100 \text{ Na}_2\text{S}_2\text{O}_3$ . The activity of the catalase is given by the difference between the quantity of the  $\text{Na}_2\text{S}_2\text{O}_3$  employed in the control and in the experiment. The results are shown in Fig. 1. When the results are plotted logarithmically as in Fig. 2, a straight line is obtained.

If we compare our results with those of Warburg *et al.* [1924], upon the course of glycolysis during embryonic growth we find a certain relationship between the two curves. In the initial stages of the embryonic development we find on the one hand the highest values of glycolysis and on the other the lowest values of catalase activity. Towards the end of development the glycolysis decreases and the catalase rises. It is certain that the catalase does not represent the only enzyme of respiration, but it can be employed as an indicator of the changes taking place in respiratory characteristics during embryonic growth.

#### SUMMARY

Figures are given for the rise in catalase content during the development of the chick embryo. The activity of the catalase is inversely related to the anaerobic glycolysis of the chick embryo. High values of catalase correspond to low degrees of glycolysis and vice versa.

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## XXXVI. STUDIES ON CHLOROPLASTS

### I. SEPARATION OF CHLOROPLASTS, A STUDY OF FACTORS AFFECTING THEIR FLOCCULATION AND THE CALCULATION OF THE CHLOROPLAST CONTENT OF LEAF TISSUE FROM CHEMICAL ANALYSIS<sup>1</sup>

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*(Received 17 December 1938)*

A NUMBER of references to experiments conducted with isolated chloroplasts are to be found in the literature. Englemann [1881] detected the evolution of oxygen by isolated chloroplasts and Ewart [1896] observed that they maintained their activity for some time after removal from the cell. Chloroplasts have been isolated from *Stellaria media* by Hill [1937] and from the leaves of the tomato plant by Granick [1937]. Noack [1927] obtained disintegrated chloroplasts by fractional centrifuging of green leaves ground with  $\text{CaCO}_3$  and Lubimenko [1929] employed autolysis to decompose cytoplasmic material. All of these workers had been concerned primarily with studies which required relatively small numbers of chloroplasts, and in most instances the details of the method of isolation were omitted.

The isolation of chloroplasts in large quantities would allow the study of their chemical structure and of the distribution of metabolites in the leaf. A study of photosynthesis by isolated chloroplasts could be carried out on a large scale and the problem of the photosynthesis of sugars by the plant attacked from this angle.

A procedure has been developed which gives a good yield of chloroplast substance suitable for chemical studies and also a slightly different procedure which gives morphologically intact chloroplasts.

After this work was completed Menke [1938] described the separation of the leaf tissue of *Spinacia oleracea* into four fractions, namely, cell wall, cytoplasm, water-soluble and chloroplast substances. This method is similar in principle to the one described herein, but is less convenient to use when only the chloroplast substance is desired. He does not give evidence for the chloroplasts being morphologically intact in his preparations. More recently Granick [1938] has described a similar method which gives morphologically intact chloroplasts.

Since the method of separating chloroplast granules depends on their flocculation, a study of the factors influencing the rate and degree of flocculation has been made. A knowledge of the percentage of the dry weight of leaf tissue which is contributed by chloroplasts is desirable in any study of the distribution of metabolites in leaf tissue. The chloroplast content of various leaves has been determined directly by weighing the chloroplast substance separated from a known amount of leaf tissue and indirectly by calculation from the analyses of

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the chloroplast substance and corresponding leaf tissue for chlorophyll, carotene and xanthophyll which are known to be present only in the chloroplasts. The results obtained are discussed with reference to the relative ease of extraction of the plastid pigments and the condition of the plastid pigments in the chloroplasts.

#### EXPERIMENTAL

Chloroplasts exhibit osmotic properties similar to those of cells. The evidence for the existence of an osmotically active membrane surrounding the chloroplasts has been reviewed by Weier [1938]. Observations, in this connexion, have been made in order to determine the effect on the chloroplasts of the medium used in their isolation.

Chloroplasts (*Trifolium pratense*) swell and disintegrate after a few minutes in distilled water. The swollen chloroplast becomes vacuolated and appears granular in structure. These granules are discoid or spherical, green in colour and have a smooth surface. They appear to be optically homogeneous chloroplasts but are about one-sixth of the size of normal chloroplasts. The granules also swell and become vacuolated in distilled water but much more slowly than chloroplasts. They may stand in distilled water for several weeks without rupturing. When chloroplasts are isolated with distilled water the material so obtained is made up chiefly of these granules, which may therefore be regarded as intact bodies representing the chlorophyllous structure of the cell. When 0.5 *M* glucose or sucrose solutions are used instead of distilled water the chloroplasts do not all disintegrate and the preparations obtained consist of intact chloroplasts as well as free granules. There is no special advantage in using these sugar solutions when one is isolating the chloroplast substance for chemical studies, the object of which is to determine the nature of the medium in which the photosynthesis of carbohydrates occurs. The analysis of these granules, which are probably the ultimate seat of photosynthesis, should provide the desired information. A method has been evolved which enables one worker to prepare 2-3 g. (dry wt. basis) of the chloroplast substance per day.

The principles on which this method is based are as follows. (a) If leaf tissue is ground in a mortar, without the addition of any other solid substance to act as a grinding agent, the cells will be ruptured, freeing the chloroplasts. (b) Starch grains are larger and denser than chloroplasts and may be separated from chloroplasts by centrifuging the suspension at a controlled speed. Only a few of the chloroplasts will be removed by this treatment. (c) Chloroplast granules in suspension may be flocculated by ions such as  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  and when flocculated they settle by gravitation.

The exact details of the method vary with the material. Four species have been employed successfully, namely *Trifolium pratense* (red clover), *Elodea canadensis*, *Arctium minus* (common burdock) and *Onoclea sensibilis* (sensitive fern). Species with fibrous leaves such as *Agropyron repens* (couch grass), or mucilaginous leaves such as the young leaves of *Tilia glabra* (basswood) do not provide suitable material. In preparing the sample as much fibrous material as possible should be removed, e.g. with red clover the three leaflets are taken by breaking at the point where they join together; with *Elodea* the bushy, green tips are taken; with burdock, the large midrib is removed from the leaf and with *Onoclea* the leaflets of the fronds are broken off about half an inch from the petiole.

After washing the leaves in distilled water and squeezing by hand to remove excess water, about 20 g. of the compressed material are sliced with scissors into

an 8 in. porcelain mortar and pounded to a pulp. About 200 ml. of distilled water are added, in three portions, and the pulp ground after each addition. This gives a suspension of free chloroplast granules in the diluted cytoplasm. Most of the fibre and unground material is next removed by filtering the suspension through bolting-silk (200-mesh) and squeezing the cloth. The suspension is now centrifuged in 250 ml. cups at 2000 r.p.m. In the case of the sensitive fern it is necessary to reduce the speed of centrifuging since the chloroplasts are larger and contain starch. The purpose of this treatment is to remove the starch grains which sediment at a much lower speed than do the chloroplasts. Some of the chloroplast granules will be carried down with the starch granules. The supernatant suspensions are decanted into a 1-litre measuring cylinder until 950 ml. are obtained, 2 M  $\text{CaCl}_2$  solution is added to the 1000 ml. mark and the contents of the cylinder mixed by inverting several times. The suspension is kept for about 30 min. when the flocculated chloroplast granules will have settled to about the 200 ml. mark. The supernatant liquid is now decanted or siphoned off; the flocculated chloroplast granules centrifuged at the same speed as before: the supernatant liquid decanted and the chloroplast granules triturated with distilled water using a glass rod with a rubber policeman. The centrifuging and washing of the chloroplast granules is repeated, usually three times, until the concentration of the flocculant is reduced to such a degree that the chloroplast granules start again to disperse. Probably only a trace of the flocculant remains at this stage. The material in the bottom of the centrifuge tube may be dried *in vacuo* at  $60^\circ$  for chemical analysis or redispersed if it is desired to determine enzymic activity. This material is a healthy green colour and appears to be fairly free from foreign material such as starch grains, nuclear material and cell wall fragments.

If intact chloroplasts are desired 0.5 M glucose can be used instead of distilled water and the chloroplasts centrifuged out at a high speed, without using a flocculating agent. This method can easily be modified to conform with the procedure of Menke [1938] for recovering cytoplasmic, water-soluble and cell wall material.

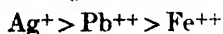
*The factors influencing the rate of flocculation of the chloroplast substance in suspension*

Many tests were carried out simultaneously, in all of which 10 ml. of the suspension of chloroplast substance, usually from red clover, were mixed with 10 ml. of a solution of the flocculant being tested. Unless otherwise stated the final concentration of the flocculant, after mixing, was always 0.1 M. The results of these experiments and the inferences drawn therefrom may be summarized as follows.

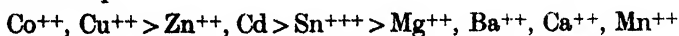
A. The rate and degree of flocculation were found to be the same with  $\text{MgCl}_2$ ,  $\text{Mg}(\text{NO}_3)_2$  and  $\text{MgSO}_4$ ; therefore the activity was due to the cation.

B. The rate of flocculation depended on the species of cation used.

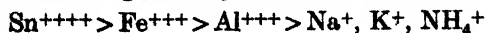
Group 1. Cations which gave a good flocculation in 1 min.



Group 2. Cations which required about 15 min. to give the same degree of flocculation as Group 1.



Group C. Cations which gave very little flocculation in 24 hr.





C. The suspensions are flocculated by hydrogen ions and the rate at which they flocculate depends, in some cases, upon the  $pH$ . When HCl or phosphate buffer solutions are used as flocculants the chloroplast substances of *Trifolium pratense* and *Arctium minus* show optimum flocculation at about  $pH$  3.7, whereas the chloroplast substances of *Onoclea sensibilis*, *Elodea canadensis* and *Solidago* sp. show no such optimum and may be flocculated with equal facility at  $pH$  5.5 and  $pH$  2.0 or even when suspended in 2*N* HCl.

D. The flocculating power of a salt solution cannot be explained as being due entirely to a favourable  $pH$ . At  $pH$  6.0 the rate of flocculation of suspensions of *Trifolium* chloroplasts decreased with decreasing concentrations of  $CaCl_2$  (0.1–0.0005 *M*). However,  $CaCl_2$  and  $FeCl_2$  at the same concentration were much better flocculants at  $pH$  3.0–3.2 than at  $pH$  4.3–4.9, whereas the flocculating power of  $AgNO_3$  was equally good in both ranges of  $pH$ .

Electrophoresis of a suspension of *Trifolium* chloroplast granules showed the chloroplast granules to be electrically charged, their mobility depending upon the  $pH$ . In 0.2*N* HCl the chloroplast substance was positively charged and in distilled water it was negatively charged. In these respects, then, the behaviour of *Trifolium* chloroplasts was analogous to that of protein molecules with an isoelectric point about  $pH$  3.7. The resemblance of the flocculation of the chloroplast substance to the precipitation of proteins was shown by Noack [1927] who observed their "salting out" by  $(NH_4)_2SO_4$ . The amphoteric nature of the chloroplast substance was not, however, the same in different plants, as it was not shown by the chloroplast substance of *Onoclea sensibilis*, which, like that of *Trifolium pratense*, contains large amounts of protein.

Flocculation by salts such as  $CaCl_2$  and  $Mg(NO_3)_2$  was reversible. If the flocculant was washed out the chloroplast substance dispersed again and the process could be repeated.  $Fe^{+++}$  and  $Al^{+++}$  were very poor flocculants whereas  $Fe^{++}$  and  $Ag^+$  were excellent flocculants. The failure of trivalent ions to cause flocculation might have been due to the fact that they were present in sufficient concentration to reverse the charge. That such was not the case was indicated by the fact that negligible flocculation was obtained with varying concentrations of  $FeCl_3$  (*M*/12–*M*/394) and  $AlCl_3$  (*M*/20–*M*/740). It was of interest to note that the chloroplast substance prepared from red clover which was growing in the autumn and had been exposed to frost, flocculated almost as well with  $AlCl_3$  as with  $CaCl_2$ . Silver was the only univalent ion which was a good flocculant. It was reduced by the ascorbic acid contained in the chloroplast. Presumably, therefore, the reduction of  $Ag^+$  removes electrons from the chloroplast substance thus reducing the negative charge and causing flocculation. The flocculating power of an ion may therefore depend on several factors such as electrical charge, age of chloroplast, solubility of a protein or lipin salt, or on some specific reaction between the ion and a constituent of the chloroplast.

#### *The calculation of the chloroplast content of leaf tissue*

The chloroplast content of leaf tissue may, theoretically, be determined in two ways: (a) by weighing the chloroplast substance separated as quantitatively as possible from a known amount of leaf tissue; (b) by analysing the chloroplasts and corresponding leaf tissue for some substance known to be found only in the chloroplasts, such as chlorophyll, carotene or xanthophyll. If, for example, 0.1 g. of leaf tissue gives a chlorophyll extract the colour of which is one-third the intensity of that derived from the same weight of chloroplast substance prepared from the same leaf tissue, then one-third of that leaf tissue is composed of chloroplasts. Obviously the first procedure will give the minimum value since

it is difficult to get all of the chloroplasts out of a given quantity of leaf tissue and since some of the chloroplast substance sediments out with the starch grains.

The values derived by different methods for the chloroplast content of the same leaf tissue are shown in Table I, and for different leaf tissues in Table II.

Table I. *Chloroplast content of red clover leaf tissue, as derived by different methods*

Method employed	Chloroplast content %
Isolation and weighing	21.7
Free chlorophyll (acetone extract)	62.1
Bound chlorophyll (acetone extract)	22.9
Carotene + xanthophyll	45.4
Carotene alone	32.2
Xanthophyll alone	50.0
Total chlorophyll (HCl treatment)	34.3

Table II. *Chloroplast content (%) of the leaf tissues of different plants, as derived by different methods*

Species	From carotene content	From chlorophyll (HCl treatment)	From xanthophyll content	From isolation and weighing
<i>Trifolium pratense</i> *	32.2	34.3	50.0	21.7
<i>Trifolium pratense</i> *	31.1	31.4	50.0	—
<i>Trifolium pratense</i> *	26.3	25.0	41.4	—
<i>Elodea canadensis</i>	16.6	36.1	10.0	30.9
<i>Onoclea sensibilis</i>	36.9	—	37.4	13.1
<i>Articum minus</i>	33.9	33.3	54.1	10.7

\* Each line represents a different preparation.

Values from carotene have an approximate error of  $\pm 2\%$ ; from chlorophyll  $\pm 1.5\%$ ; and from xanthophyll  $\pm 4\%$ .

The terms "free" and "bound" chlorophyll require some explanation. A chloroplast preparation was suspended in an 85% solution of acetone in a Büchner funnel fitted with filter paper; after a few minutes suction was applied. The extraction of chlorophyll with 85% acetone was repeated until the extract was colourless, and then the residue was extracted repeatedly and in the same manner with 10% trichloroacetic acid. A yellow pigment was removed by this treatment. If the trichloroacetic acid extraction were now followed by further extractions with 85% acetone the extracts contained considerable quantities of green pigment. The pigment thus extracted showed the typical red fluorescence of chlorophyll dissolved in acetone; it was found in whole leaf tissue and the chloroplast substance and was designated as "bound" chlorophyll.

Total chlorophyll was determined by treatment with HCl as follows: 0.1 g. portions of dried and thoroughly ground tissue were weighed into 50 ml. centrifuge bottles and extracted with 10 ml. of water by heating in a boiling water bath for about 10 min., centrifuging and decanting the supernatant liquid. This removed a brown pigment from the whole leaf tissue which interfered with the determinations. After the removal of this pigment the remainder of the procedure was carried out, simultaneously and in identical manner, with an equal weight of chloroplasts and leaf tissue, as follows: 10 ml. of 6N HCl were added, the suspension was heated on a boiling water bath for a few min., centrifuged and the supernatant liquid containing the green-coloured products of chlorophyll hydrolysis was decanted through filter paper into a 25 ml. glass-stoppered

graduated cylinder. The extraction was repeated using 5 ml. of HCl, each time, until all the green pigment had been extracted. The combined extracts were mixed and the colour of the extract from the chloroplasts compared with that from the corresponding leaf tissue. The more intensely coloured extract was diluted until, on visual comparison, the colours of the two solutions appeared to be of equal intensity. The relative volumes were noted from which the chloroplast content of the leaf tissue was calculated.

Carotene and xanthophyll determinations were carried out by weighing 0.1 g. of the dried and ground tissue into a 50 ml. centrifuge bottle and adding 3 ml. of 30% KOH in absolute methyl alcohol and 20 ml. of light petroleum. The bottles were then stoppered and shaken for 5 min. in a mechanical shaker, centrifuged and the supernatant liquid siphoned off into separating funnels. The process was repeated, until no more pigment was extracted, using 10 ml. of light petroleum and 1 ml. of alcoholic KOH. The addition of a small amount of water was sometimes helpful. The carotene and xanthophyll contained in the combined petroleum extracts were separated by extraction with 85% methyl alcohol. The carotene content of the petroleum solution and the xanthophyll content of the methyl alcohol solution were determined by using an Evelyn photoelectric colorimeter and referring to a standard calibration curve prepared from pure  $\beta$ -carotene.

The results obtained from the chlorophyll determinations by HCl treatment and from the carotene determinations compare well and appear to represent the true values (see Table II). The only exception is *Elodea canadensis* where the value derived from carotene is obviously wrong, being lower than the figure obtained by isolation and weighing. The latter values are always low but not to the same extent with different species. A smaller fraction of the total number of chloroplasts is obtained in species with fibrous leaves, such as *Arctium* and *Onoclea* than in species with non-fibrous leaves such as *Elodea*. The chloroplast content of *Onoclea sensibilis* could not be estimated from chlorophyll determinations by HCl treatment because of the presence in the extract of the whole leaf tissue of a pink colour which interfered with the colorimetry.

The values shown in Tables I and II indicate the difficulties to be encountered in the extraction of the plastid pigments. If, for example, a value higher than the true value for the chloroplast content is obtained, as is the case in the calculation of the chloroplast content of the leaves of *Trifolium pratense* from xanthophyll determinations, then this means that relatively more xanthophyll has been extracted from the whole leaf than from the chloroplast substance. If the value is too low, as in the case of *Elodea canadensis*, then the reverse holds. The results in Table I show that chlorophyll and xanthophyll are extracted with difficulty from *Trifolium* chloroplast substance by 85% acetone. Carotene is apparently extracted with equal facility from the chloroplast substance and whole leaf tissue. Both carotene and xanthophyll are more easily extracted from the chloroplast substance of *Elodea* than from the whole leaf tissue, etc. Since the green chlorophyll derivatives obtained by the HCl treatment are extracted with equal ease from the chloroplast substance and whole leaf tissue in all cases, this appears to be the most dependable method to employ.

The differences in the ease of extraction of the plastid pigments from the chloroplasts as compared with the whole leaf tissue of the same species and as contrasted with other species, can apparently be explained only by assuming the presence of other concomitant substances which condition their solubility. This view was held by Willstätter. According to Lubimenko [1929] chlorophyll *a* and *b*, carotene and xanthophylls exist in the leaf in combination with protein in the

form of a single complex pigment molecule which shows slight variations in complexity in different species. We have observed that a purified chlorophyll preparation on treatment with HCl in the manner already described yields no green-coloured derivatives and that the addition of this chlorophyll preparation to chloroplasts does not increase the amount of green pigment extracted with HCl over that obtained from the chloroplasts alone. These observations are interpreted as showing that chlorophyll, as it exists in the chloroplast, perhaps in the complex pigment of Lubimenko, is decomposed by HCl treatment in a different manner from chlorophyll which has been extracted by acetone and freed from carotenoid pigments.

#### SUMMARY

A method which enables one worker to isolate chloroplasts from leaf tissue at the rate of 2-3 g. daily has been described.

The chloroplast substance in suspension is charged; the charge depends on the pH being negative above pH 3.7. Various cations cause the flocculation of these suspensions. This flocculation cannot be explained as a simple electrical neutralization of charges; evidently specific reactions may occur between the chloroplast substance and certain cations which are responsible for the anomalies noted. The age of the chloroplast may have some effect.

Several methods for calculating the chloroplast content of leaf tissue have been worked out and compared. These methods are based on analysis for the plastid pigments. The results obtained show that completeness of extraction of these pigments is affected by the presence of unknown substances. A simple method based on analysis for chlorophyll gives satisfactory results in most cases.

I wish to express my thanks to Dr W. D. McFarlane for the facilities extended, for helpful criticism and for assistance in preparing the manuscript.

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# XXXVII. STUDIES ON CHLOROPLASTS

## II. THEIR CHEMICAL COMPOSITION AND THE DISTRIBUTION OF CERTAIN METABOLITES BETWEEN THE CHLOROPLASTS AND THE REMAINDER OF THE LEAF<sup>1</sup>

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THE available information concerning the chemical composition of the chloroplasts and the distribution of elements within the cell has been gained by the use of histochemical methods. The studies reported in this paper were made on the chloroplast substance isolated by the method outlined in the previous paper [1939]. Some information on the composition of the chloroplasts isolated from the leaves of two different species was first obtained. The distribution of N, P, Mg, Fe and Ca in the chloroplasts was next investigated by means of various solvents to obtain information on the nature of the combination of these elements. To determine which elements or compounds were concentrated in the chloroplasts, comparative analyses were made of chloroplast preparations and the corresponding whole leaf tissue; these comprised determinations of Ca, Fe, Cu, P, Mg, K, Na, Mn, SO<sub>4</sub>, Cl, NH<sub>4</sub> salts, nitrates, ascorbic acid, catalase and carbonic anhydrase in the chloroplasts and whole leaf tissue of four different species of plants.

### EXPERIMENTAL

Analyses were made of the whole leaf tissue and of the chloroplast substance separated from the leaf tissues of *Trifolium pratense* and *Onoclea sensibilis*. The samples were dried *in vacuo* at 60°. The protein content was estimated by determining the N content of the residue remaining after exhaustive extraction of the material with 85 % acetone, absolute alcohol, anhydrous ether, 10 % trichloroacetic acid and 85 % acetone in the order named, and multiplying the value obtained by 6.25. The difference between the total N and the protein-N is represented as the non-protein-N. The ether extract was determined by continuous extraction of the original material in a Soxhlet apparatus with ether only. Starch was determined by the taka-diastase method of Denny [1934] and ammonia by the method of Pucher *et al.* [1935]. In determining nitrates, 0.20 g. of the material was extracted first with ether and then with boiling water; the extracts were combined and freed from ether. The aqueous extract was next made slightly alkaline and evaporated to dryness; the residue was taken up in water and brought to 200 ml. Nitrates were determined in this solution by means of the colorimetric diphenylbenzidine method of Whelan [1930].

Ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol according to McHenry & Graham [1935]. The titrations were carried out in triplicate with suspensions of freshly prepared chloroplasts or ground leaf

<sup>1</sup> Macdonald College Journal; Series No. 109.

tissue, in the preparation of which a few drops of 10% KCN were added to prevent oxidation of ascorbic acid. The suspensions were saturated with  $\text{H}_2\text{S}$  and kept overnight in a closed vessel. The  $\text{H}_2\text{S}$  was displaced by  $\text{CO}_2$ , the nitroprusside test being employed to determine when all the  $\text{H}_2\text{S}$  had been removed. The titration was carried out in a centrifuge tube with the least possible delay. A few ml. of 2 M  $\text{CaCl}_2$  were added to ensure the flocculation of the suspension, more of the dye solution was added and the suspension centrifuged until the end-point of the titration was reached, which could be easily detected in the clear supernatant liquid. The first titration gives an approximate value; the second and third titrations can be conducted more accurately.

Carotenoid pigments were determined as previously described [1939]. P was determined according to Dyer & Wrenshall [1938]; Fe and Cu by the methods of Parker & Griffin [1939]; Ca by the volumetric micromethod of the A.O.A.C. [1935]; Mg by the hydroxyquinoline method of Greenberg *et al.* [1935]; Na by the method of Salit [1932]; K by the volumetric cobaltinitrite method of Volk & Truog [1934]; Mn by the colorimetric bismuthate method of the A.O.A.C. [1935];  $\text{SO}_4$  by the volumetric benzidine method of Cope [1931] and Cl by the method of Cattle [1935].

The catalase and carbonic anhydrase activities of suspensions of the chloroplast substance and ground whole leaf tissue in distilled water were determined with a differential manometer. It was necessary to use the thermostat at a temperature of  $27^\circ$  because of the high atmospheric temperature prevailing at the time. The amount of gas evolved was plotted against time for suspensions of known dilution and in this way the time was found at which the amount of gas evolved is a measure of the enzymic activity.

In the fractionation of the chloroplasts a 2 g. sample of the moisture-free, finely pulverized chloroplasts was employed in each case. The extractions with anhydrous solvents were made in a Soxhlet extractor, but with the other solvents repeated extractions were carried out in a Büchner funnel fitted with filter paper.

Table I. *Analysis of the chloroplast substance of Trifolium pratense and Onoclea sensibilis*

Results expressed as % of the moisture-free substance

	<i>Trifolium pratense</i>	<i>Onoclea sensibilis</i>
Total N	9.69	5.90
$\text{NH}_4\text{-N}$	0.80	0.12
$\text{NO}_3\text{-N}$	0.011	0.006
Non-protein-N	1.66	0.87
Protein-N	8.02	5.05
Total protein	50.11	31.51
Ether extract	21.75	7.21
Starch	Nil	8.43
Ascorbic acid	2.53	0.56
Carotene	0.19	0.10
Xanthophylls	0.24	0.15
Total ash	3.02	4.11
Mg	0.166 : 0.164	0.113 : 0.164
Cu	0.008	0.017
Fe	0.092	0.074
Ca	0.170	0.220
P	0.219 : 0.741	0.326 : 0.254
K	Nil	Nil
Na	0.039	0.939
Mn	Trace	Trace
$\text{SO}_4$	0.894	0.431
Cl	Nil	0.074

## RESULTS AND DISCUSSION

1. *Composition of chloroplasts*

The results are shown in Table I. The high protein and lipid contents of the chloroplasts, particularly of *Trifolium pratense*, are noteworthy. These results agree as well as could be expected with those of Menke [1938] who records 8.38 % N and 30.9 % ether extract for the chloroplasts of *Spinacia oleracea*. The chloroplasts of *Onoclea sensibilis* are different from those of the other two species; they are considerably larger and appear to store more starch than those of clover, which accounts, in part at least, for the lower lipid and protein contents. It will also be noted that the P content varied markedly in two preparations from the leaves of the same species cut at different stages of growth.

2. *Fractionation of chloroplasts*

In Table II are shown the scheme of extraction followed and the results obtained. When the initial extraction was made with 85 % acetone more material was dissolved than when it was made with anhydrous ether (Table II B). Furthermore, extraction with 85 % acetone left only a small fraction which was extractable by alcohol and ether, so that most of the lipid material of the chloroplast was extracted by 85 % acetone. An appreciable amount of P was extracted by anhydrous ether but when the material was first extracted with 85 % acetone the amount of P extracted by ether was considerably reduced. It would appear, therefore, that the chloroplasts contain phospholipins which are appreciably soluble in 85 % acetone. Granick [1937] found that 40 % of the total lipid-P of the tomato leaf was located in the chloroplasts. Most of the P in clover chloroplasts was extracted by trichloroacetic acid, whereas the P in the chloroplasts of *Onoclea sensibilis* was found to be more difficult to extract.

Nearly all of the Mg contained in the chloroplasts was extracted by trichloroacetic acid, and when compared with this fraction the amount of Mg present in the chlorophyll fraction was negligible. Ca was also nearly all removed by the trichloroacetic acid. This is in accord with the findings of Terlikowski and Sozonski [1937] and Kostychev & Berg [1929], that extraction of leaf tissue with dilute HCl acid effects almost complete removal of the Ca and Mg.

A considerable proportion of the total Fe was extracted by trichloroacetic acid and an appreciable amount was also dissolved by previous extraction with ether, which is to be expected from the known solubility of inorganic salts of Fe in ether. Even after extraction with trichloroacetic acid, Fe was found in a subsequent 85 % acetone extract. The Fe contained in the chloroplasts of *Onoclea sensibilis* seems to be in a form readily soluble in acetone which may account for the comparatively small amount contained in the trichloroacetic acid fraction. The nature of the combination of Fe in the chloroplast is discussed later. The ionic state of the Ca, Mg, P and Fe in the trichloroacetic acid extracts was shown by the fact that the amounts of these elements, as obtained by direct analysis of the trichloroacetic acid solution, were nearly equal to the amounts obtained after digestion with conc.  $\text{H}_2\text{SO}_4$  plus  $\text{Na}_2\text{SO}_4$  and  $\text{HgO}$ .

Most of the N remains in the final residue and is probably all protein-N. Small amounts are extracted by trichloroacetic acid or ether, that dissolved by the latter probably being contributed by lecithin. Granick [1938] found that 80 % of the total N in chloroplasts was protein-N. This agrees with the values reported here.

Table II. *Showing the analysis of various fractions separated from the chloroplasts of Trifolium pratense and Onoclea sensibilis*

Results expressed as % of the total amount in the original material

## A. Dry, powdered, chloroplast substance extracted with anhydrous ether

Residue (Extracted with absolute alcohol, followed by absolute acetone)					
Residue (Extracted with 80% acetone, followed by extraction with 10% trichloroacetic acid)					
Ether extract			Acetone extract		Trichloroacetic acid extract
	<i>Trifolium</i>	<i>Onoclea</i>	<i>Trifolium</i>	<i>Onoclea</i>	<i>Trifolium</i> <i>Onoclea</i>
Dry wt.	21.75	7.21	0.55	0.90	3.35   0.42
N	2.26	0.73	0.18	0.93	67.97   14.12
P	3.10	1.13	*	0.015	90.85   78.76
Mg	*	9.74	*	*	66.96   54.21
Fe	6.11	*	3.49	6.52	92.12   103.64

## B. Dry, powdered, chloroplast substance extracted with 85% acetone

Soluble in 85% acetone (Residue extracted with light petroleum)					
Residue (Extracted with absolute alcohol and anhydrous ether)					
Extract			Extract		
	<i>Trifolium</i>	<i>Onoclea</i>	<i>Trifolium</i>	<i>Onoclea</i>	
Dry wt.	25.14	12.24	3.75	1.53	
N	5.86	1.37	0.32	0.32	
P	1.42	6.90	0.23	0.64	
Mg	*	*	*	*	
Fe	*	*	1.53	2.44	
Residue (Extracted with 10% trichloroacetic acid)					
Residue (Extracted with 80% acetone)					
Extract			Extract (bound chlorophyll)		Residue
	<i>Trifolium</i>	<i>Onoclea</i>	<i>Trifolium</i>	<i>Onoclea</i>	<i>Trifolium</i> <i>Onoclea</i>
N	3.18	0.32	3.03	0.63	82.73   85.51
P	55.43	19.31	3.88	11.57	—   —
Mg	87.80	61.95	*	*	—   —
Fe	47.76	26.49	2.29	7.74	—   —
Ca	92.12	83.62	—	—	—   —

\* If present, the amount found was too small to be determined by the methods employed.



## 3. Comparative analysis of chloroplasts and whole leaf tissue

The results of the analysis of the whole leaf tissue of different species of plants and of the chloroplast substance separated from the same leaf tissue are shown in Tables III, IV and VII. When reported as % of the total ash

Table III. Showing the comparative compositions of the ash of chloroplasts and whole leaf tissue

Species	Total ash (% dry wt.)	% of the total ash									
		Fe	Ca	Mg	P	Mn	K	Na	Cu	SO <sub>4</sub>	Cl
<i>Trifolium pratense:</i>											
Chloroplast substance	8.04	0.44	6.15	1.99	9.20	—	*	0.48	0.101	11.11	*
Whole leaf	9.91	0.27	20.87	3.08	3.84	—	20.01	3.21	0.042	7.31	1.11
<i>Onoclea sensibilis:</i>											
Chloroplast substance	5.23	1.08	6.33	3.04	4.90	—	*	0.74	0.332	8.23	2.12
Whole leaf	9.75	0.44	13.31	5.12	3.80	0.06	21.01	2.14	0.077	10.58	5.63
<i>Elodea canadensis:</i>											
Chloroplast substance	9.74	2.56	—	2.07	4.01	0.24	*	2.57	0.165	6.89	—
Whole leaf	12.89	1.14	—	3.41	3.56	0.52	25.28	5.16	0.068	6.21	—
<i>Arctium minus:</i>											
Chloroplast substance	5.32	1.18	9.62	3.00	6.39	—	*	0.39	0.160	8.09	0.74
Whole leaf	11.75	0.59	14.02	4.86	2.64	—	21.68	3.35	0.031	5.21	3.54

\* Presence doubtful—not detectable by the methods used.

Table IV. Showing the distribution of ammonia, nitrates, sulphates and ascorbic acid in leaf tissue

Results are expressed as % of the moisture-free substance

Species	Ammonia (% NH <sub>4</sub> -N)			Nitrates (% NO <sub>3</sub> -N)		
	Chloro- plasts	Whole leaf	Non- chloroplast fraction	Chloro- plasts	Whole leaf	Non- chloroplast fraction
<i>Trifolium pratense</i>	0.794	0.335	0.165	0.0110	0.0062	0.0045
<i>Onoclea sensibilis</i>	0.125	0.088	0.066	0.0060	0.0034	0.0051
<i>Elodea canadensis</i>	0.234	0.110	0.033	0.0006	0.0044	0.0073
<i>Arctium minus</i>	0.217	0.172	0.149	0.0032	0.0156	0.0208
Sulphates						
	Chloro- plasts	Whole leaf	Non- chloroplast fraction	Chloro- plasts	Whole leaf	Non- chloroplast fraction
<i>Trifolium pratense</i>	0.89	0.66	0.57	2.529	2.557	2.566
<i>Onoclea sensibilis</i>	0.43	1.03	1.38	0.566	0.285	0.128
<i>Elodea canadensis</i>	0.67	0.80	0.88	0.653	0.433	0.325
<i>Arctium minus</i>	0.43	0.61	0.70	0.497	0.677	0.770

(Table III), or as % of the total amount in the whole leaf which is contained in the chloroplast fraction (Table V), the values show which elements or compounds are concentrated in the chloroplasts relative to the other fractions. The whole leaf material is always higher in total ash content than the chloroplast substance. From these results the following observations may be made.

A. Fe and Cu are concentrated in the chloroplasts, Cu showing an even greater tendency to collect in the chloroplasts than Fe. The fact that Fe and Cu show the same localization supports the view that some oxidative reactions are centred in the chloroplasts which particularly require their catalytic action.

Table V. *Showing the distribution of elements between the chloroplasts and the remainder of the leaf tissue*

Results are expressed as % of the moisture-free substance

	<i>Trifolium pratense</i>	<i>Onoclea sensibilis</i>	<i>Elodea canadensis</i>	<i>Arctium minus</i>
% of chloroplasts in the leaf tissue	27	37	40	33
Fe: Whole leaf	0.025	0.043	0.147	0.070
Chloroplast substance	0.035	0.057	0.249	0.063
Non-chloroplast fraction	0.022	0.035	0.092	0.077
Ca: Whole leaf	1.91	1.43	—	2.45
Chloroplast substance	0.17	0.22	—	0.72
Non-chloroplast fraction	2.55	2.14	—	3.29
Mg: Whole leaf	0.28	0.50	0.44	0.57
Chloroplast substance	0.16	0.16	0.20	0.12
Non-chloroplast fraction	0.33	0.70	0.60	0.78
P: Whole leaf	0.35	0.37	0.46	0.31
Chloroplast substance	0.74	0.26	0.39	0.34
Non-chloroplast fraction	0.21	0.44	0.50	0.29
K: Whole leaf	1.82	2.05	3.26	2.54
Chloroplast substance	*	*	*	*
Non-chloroplast tissue	2.49	3.25	5.43	3.79
Na: Whole leaf	0.29	0.21	0.67	0.39
Chloroplast substance	0.04	0.04	0.25	0.02
Non-chloroplast tissue	0.38	0.32	0.95	0.07
Cu: Whole leaf	0.0038	0.0025	0.0087	0.0038
Chloroplast substance	0.0081	0.0174	0.0160	0.0085
Non-chloroplast fraction	0.0022	0.0017	0.0038	0.0009
Cl: Whole leaf	0.10	0.61	—	0.62
Chloroplast substance	*	0.07	—	0.05
Non-chloroplast fraction	0.14	0.94	—	0.90
Mn: Whole leaf	Trace	0.01	0.07	Trace
Chloroplast substance	Trace	Trace	0.02	Trace
Non-chloroplast fraction	—	—	0.10	—

\* Presence doubtful—not detected by methods used.

Determinations of inorganic Fe and Cu showed that whilst most of the Fe in the chloroplasts is present in the inorganic form there is an appreciable amount which may be in organic combination, and that Cu is present in the chloroplasts largely in organic combination (Table VI).

Table VI. *Nature of the combination of Fe and Cu in the chloroplasts and whole leaf tissue of Trifolium pratense*

Fe (mg. per g. dry tissue):	Chloroplasts	Whole leaf
Total	0.772	0.479
In 10% acetic acid extract	0.572	0.292
Inorganic Fe (% of total Fe)	74.1	61.0
% of the Fe in the whole leaf which is contained in the chloroplast fraction: (a) inorganic, 51.7%; (b) organic, 57.5%.		
Cu (mg. per g. dry tissue):		
Total	0.081	0.038
In 10% acetic acid extract	Nil	0.004
Inorganic Cu (% of total Cu)	—	10.5
% of the total Cu in the whole leaf which is contained in the chloroplast fraction: 74.6%.		

B. *Ca* and *Mg* show a similar localization, being found mainly in the cytoplasm, although the chloroplasts do contain appreciable amounts of these elements. This distribution is interesting from the viewpoint of Laville [1933] who believes that *Mg* antagonizes *Fe* by its antioxidant activity; the two elements show opposite localizations in the leaf thus allowing each other greater freedom of action.

C. *P*, relative to the other constituents of the ash, is concentrated in the chloroplasts; the degree of concentration, however, is variable. Granick [1937] has recorded that 20 % of the total *P* and 40 % of the lipoid *P* in tomato leaves is contained in the chloroplasts. This does not show an accumulation of *P* in the chloroplasts unless the chloroplast content of this leaf is very small. That the *P* content of the chloroplasts of *Trifolium pratense* may vary widely is shown from the two results given in Table I, which represent the *P* contents of the chloroplasts of the young growing plant and of the flowering plant respectively. According to Seissl [1909] the *P* content of the leaves is at a maximum during the period of greatest  $\text{CO}_2$  assimilation.

D. *Na* and *K* are concentrated outside of the chloroplast; *K* is probably absent from the chloroplast. These observations are in agreement with the findings of Weevers [1913] and Lloyd [1925], by histochemical tests, that *K* was localized in the vacuoles and of Funcoka [1921] that *Na*, by similar tests, is localized in the cell plasma.

E. *Mn* appears to be present in both the chloroplasts and non-chloroplast material of the leaf and particularly in the latter. In some cases there is only a trace of *Mn* present.

F.  $\text{SO}_4$  and *Cl* show a variable distribution in the leaves of different species.  $\text{SO}_4$  in particular shows no evidence of a definite localization, whereas *Cl* appears to be concentrated outside the chloroplast, presumably as a result of the high concentration of potassium in the cell sap (see Table IV).

G.  $\text{NH}_4$  salts appear to be more highly concentrated in the chloroplasts, whereas nitrates, like sulphates, do not show any definite plan of localization.

H. *Ascorbic acid* appears to be present in appreciable quantities both in the chloroplasts and in the other parts of the cell. This is in accord with the findings, by histochemical tests of Giroud *et al.* [1934; 1935] and of Dischendorfer [1936] who conclude further, that there is a positive correlation between the amount of chlorophyll and ascorbic acid present and that the latter is bound in the chloroplasts.

I. *Catalase* and *carbonic anhydrase* are found in the chloroplasts, the former being highly concentrated in the chloroplasts, in all of the species. Carbonic anhydrase is also present in the other parts of the leaf in amounts varying with the species. The values for the whole leaf tissue are a little too low from the fact that it was difficult to rupture all the cells by grinding. This explains why the values in Table VII show a higher localization of catalase in the chloroplasts than is theoretically possible. However, at least 80–90 % of the cells should be ruptured since 10 g. of the material were ground as thoroughly as possible in a large porcelain mortar. Von Euler and his associates [1929; 1930] have found a relationship between the chlorophyll content and catalase activity, and between the number of chloroplasts present and catalase activity, which appeared to be hereditary.

It is interesting to note that *Fe*, *Cu* and catalase, which are concentrated in the chloroplasts, are oxidative catalysts. This would indicate that active respiratory processes occur in the chloroplasts.

Table VII. *Showing the distribution of carbonic anhydrase and catalase in leaf tissue*

	<i>Trifolium pratense</i>		<i>Arctium minus</i>		<i>Onoclea sensibilis</i>	
	Carbonic anhydrase	Catalase	Carbonic anhydrase	Catalase	Carbonic anhydrase	Catalase
Activity of chloroplast* substance	13.89	7.06	57.90	12.12	50.04	13.63
Activity of whole leaf*	15.37	1.78	24.84	3.83	38.22	4.78
Activity of whole leaf due to chloroplasts (calculated)	3.75	1.90	19.30	4.30	18.51	5.04
% of total activity of leaf which is due to chloroplasts	24.39	106.74	77.69	112.27	48.43	105.43

\* Carbonic anhydrase activities are represented in  $\mu\text{l. CO}_2$  released per mg. of dry tissue in 30 sec. at 27°.

Catalase activities are represented in  $\mu\text{l. O}_2$  released from 0.01 *M*  $\text{H}_2\text{O}_2$  per mg. of dry tissue in 2 min. at 27°.

## SUMMARY

Chloroplasts consist chiefly of protein and lipins. They contain a relatively high percentage of lipins as compared with the rest of the cell. Nearly all the lipin fraction may be extracted with 85 % acetone.

Cu, Fe, P and  $\text{NH}_4$  salts are concentrated to a certain extent in the chloroplasts. Ca, Mg, Mn, Na, K and Cl show an opposite localization in the cell.  $\text{SO}_4$  and  $\text{NO}_3$  do not follow any general rule.

The Cu in chloroplasts appears to exist chiefly in organic combination. Part of the Fe and P is also combined organically but Ca and Mg are present chiefly in the inorganic state.

Most of the catalase in the leaf cells is found in the chloroplasts. Carbonic anhydrase and ascorbic acid are found in appreciable quantities both in the chloroplasts and in other parts of the cells.

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# XXXVIII. INTERACTION OF VITAMIN C AND TISSUE PHOSPHATASES

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In previous publications [Giri, 1938, 1 and 2] the results of experiments on the interaction of plant phosphatases, vitamin C and glutathione were reported. A similar investigation on animal tissue phosphatases has been undertaken with a view to completing a general survey of the effect of vitamin C upon the activity of phosphatases of both plant and animal origin.

## EXPERIMENTAL

*Preparation of material.* The tissues used in the present investigation were those of sheep kidney, liver and brain. Dry powders were prepared from the tissues by treatment with acetone and ether as described by Giri & Datta [1936]. Active extracts were obtained from the acetone-treated tissue materials, as described below.

The method of determining the activity of the phosphatase and the other procedures were similar to those already described [Giri, 1938, 2].

### I. KIDNEY PHOSPHATASE

*Influence of vitamin C on the activity of kidney phosphatase.* 10 g. acetone-treated kidney powder were extracted with 100 ml. water, saturated with

Table I. *Influence of vitamin C on the activity of kidney phosphatases  
without purification by dialysis*

*Reaction mixture.* 10 ml. glycine buffer *M*/5 (pH 8.9) (for alkaline phosphatase) and 10 ml. *M*/5 acetate buffer (pH 5.0); 10 ml. 2% sodium- $\beta$ -glycerophosphate (Merck); 4 ml. 0.1 *M* MgCl<sub>2</sub>, 6H<sub>2</sub>O (only for alkaline phosphatase); 5 ml. enzyme extract. Total volume, 40 ml.; vitamin C, 5 mg.; Cu as CuSO<sub>4</sub>, 5H<sub>2</sub>O, 0.062 mg.

Time of hydrolysis in min. ...	Activity in mg. in 10 ml. reaction mixture	
	Alkaline phosphatase	Acid phosphatase
	60	120
Crude phosphatase	0.642	0.130
Crude phosphatase + vitamin C	0.642	0.130
Crude phosphatase + vitamin C + Cu	0.620	0.130
Crude phosphatase + Cu	0.642	0.130
Oxidation of vitamin C		
	mg. of vitamin C in the total volume of reaction mixture	
Crude phosphatase + vitamin C	4.6	4.4
Crude phosphatase + vitamin C + Cu	2.0	3.5

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toluene for 24 hr. and filtered. A portion of the filtrate (30 ml.) was dialysed for 2 days in collodion bags and centrifuged to remove the suspended impurities.

The results show that the vitamin C, Cu and the vitamin C-Cu complex have no influence on the activity of the alkaline and acid phosphatases of kidney when crude aqueous extracts without purification by dialysis are used (Table I).

Table II A. *Influence of vitamin C on the activity of kidney phosphatase purified by dialysis*

*Reaction mixture.* 10 ml. glycine buffer *M*/5 (*pH* 8.9) for alkaline phosphatase and 10 ml. acetate buffer *M*/5 (*pH* 5.0) for acid phosphatase; 10 ml. 2% Na- $\beta$ -glycerophosphate; 4 ml. enzyme extract; total volume, 40 ml.; vitamin C, 5 mg.; Cu as CuSO<sub>4</sub>, 5H<sub>2</sub>O (0.082 mg.).

Alkaline phosphatase				
Activity in mg. P in 10 ml. reaction mixture				
Time of hydrolysis in min. ...	15	30	60	
1. Purified phosphatase	0.470	0.800	1.50	
2. Purified phosphatase + vitamin C	0.470	0.800	1.50	
3. Purified phosphatase + vitamin C + Cu	0.300	0.500	1.08	
4. Purified phosphatase + Cu	0.470	0.800	1.50	

Oxidation of vitamin C				
mg. vitamin C in the total volume of reaction mixture				
Time in min. ...	0	15	30	60
2. Purified phosphatase + vitamin C	5.0	4.9	4.8	4.5
3. Purified phosphatase + vitamin C + Cu	5.0	2.8	2.4	1.3

Table II B.

Acid phosphatase				
Activity in mg. P in 10 ml. reaction mixture				
Time of hydrolysis in min. ...	60	120		
1. Purified phosphatase	0.083	0.154		
2. Purified phosphatase + vitamin C	0.083	0.150		
3. Purified phosphatase + vitamin C + Cu	0.066	0.100		
4. Purified phosphatase + Cu	0.083	0.150		

Oxidation of vitamin C				
mg. vitamin C in the total volume of reaction mixture				
Time in min. ...	0	30	60	120
1. Purified phosphatase + vitamin C	5.0	4.9	4.7	4.7
3. Purified phosphatase + vitamin C + Cu	5.0	4.8	2.6	1.3

It can be seen from the figures in Tables II A and B, that the vitamin C-Cu complex inhibits the activity of the purified phosphatase, while vitamin C or copper alone has no influence. Thus the behaviour of the phosphatase present in dialysed aqueous extracts of kidney towards the vitamin C-Cu complex is different from that of the enzyme present in crude aqueous extracts of the tissue. Some factors present in the latter appear to interfere with the inhibition of the activity of the phosphatase by the vitamin C-Cu complex. In the light of the previous observations made on the influence of vitamin C on plant phosphatases, it was thought that the factors which interfere with the inhibition of the activity of the phosphatase by the vitamin C-Cu complex, protect the vitamin against oxidation, thereby preventing the inhibition. The following experiment

was therefore undertaken with a view to finding out whether the protective action of kidney extracts against the oxidation of the vitamin is responsible for the differences observed in the behaviour of crude aqueous extracts and dialysed extracts of kidney towards the vitamin C-Cu complex (Table III).

Table III. *Protective action of dialysed and undialysed kidney extracts against the oxidation of vitamin C*

*Reaction mixture.* 20 ml. acetate buffer  $M/5$  ( $pH$  5.0); 5 ml. extract; 5 mg. vitamin C; Cu as  $CuSO_4 \cdot 5H_2O$  (0.062 mg.); total volume, 40 ml.

	Time in min. ...	mg. vitamin C in the total volume of reaction mixture			
		0	15	30	60
1. Vitamin C	5.0	5.0	3.4	3.0	2.3
2. Vitamin C + dialysed extract	5.0	5.0	3.8	3.4	3.0
3. Vitamin C + undialysed extract	5.0	5.0	5.0	5.0	5.0
4. Vitamin C + Cu	5.0	5.0	2.7	2.5	1.4
5. Vitamin C + Cu + dialysed extract	5.0	5.0	2.5	2.4	1.0
6. Vitamin C + Cu + undialysed extract	5.0	5.0	4.5	4.1	3.6

It is seen from these results that undialysed extracts contain some substances which protect the vitamin against catalytic oxidation by Cu, while the dialysed extract does not exert any such protective action, although a slight protection in the absence of Cu is noticed. Further, the dialysed extracts contain certain substances which accelerate the oxidation of vitamin C by Cu. Such constituents, which are probably colloidal in nature, are found to be present in the dialysed extracts of acetone-treated liver and brain. Further investigation of the nature of the constituents and their relation to the oxidation of the vitamin is in progress.

The results so far obtained on the influence of the vitamin C-Cu complex on the activity of kidney phosphatase show that the activity is in some way related to the oxidation of the vitamin.

## II. LIVER PHOSPHATASE

### *The influence of vitamin C on the activity of liver phosphatase*

*Preparation of active extract.* 20 g. acetone-treated liver powder were extracted with 100 ml. water saturated with toluene for 48 hr. at room temperature and filtered. The filtrate, which was coloured yellow, was used directly. The dialysed extract was prepared by dialysing the crude aqueous extract (60 ml.) for 3 days and centrifuging to remove the suspended impurities.

The results are similar to those obtained with kidney phosphatase. The liver phosphatase activity is inhibited by the vitamin C-Cu complex when dialysed extracts are used as the source of the enzyme, while the activity of the phosphatase present in crude aqueous extracts is not affected by vitamin C-Cu complex. Neither vitamin C nor copper alone has any influence on the activity (Table IV).

*Protective action of liver extracts against the oxidation of vitamin C.* Table V shows the difference in the protective properties of the dialysed and undialysed extracts of acetone-treated liver powder.

It can be seen from the results that undialysed extracts of liver protect the vitamin against catalytic oxidation, while the dialysed extracts lack such protective properties. The difference in behaviour of liver phosphatase contained in dialysed and undialysed extracts towards the vitamin C-Cu complex



Table IV. *Influence of vitamin C on the phosphatase activity of dialysed and undialysed liver extracts*

*Reaction mixture.* 10 ml. glycine buffer *M*/5 (pH 8.9); 10 ml. 2% Na- $\beta$ -glycerophosphate; 4 ml. 0.1 *M* MgCl<sub>2</sub>, 6H<sub>2</sub>O (only for alkaline phosphatase); 5 ml. enzyme extract; total volume, 40 ml. Vitamin C, 5 mg.; Cu as CuSO<sub>4</sub>, 5H<sub>2</sub>O (0.062 mg.). Temperature, 35  $\pm$  0.1°.

Time of hydrolysis ...	Activity in mg. P in 10 ml. reaction mixture			
	Undialysed extract	Dialysed extract		
		1 hr.	3 hr.	5 hr.
1. Phosphatase	0.160		0.690	1.00
2. Phosphatase + vitamin C	0.160		0.680	0.950
3. Phosphatase + vitamin C + Cu	0.160		0.520	0.714
4. Phosphatase + Cu	0.160		0.690	1.00

## Oxidation of vitamin C

Time in min. ...	mg. vitamin C in the total of reaction mixture							
	Undialysed extract				Dialysed extract			
	0	30	60		0	30	60	120
2. Phosphatase + vitamin C	5.0	4.5	4.1		5.0	4.6	4.3	3.3
3. Phosphatase + vitamin C + Cu	5.0	3.8	3.3		5.0	2.2	1.5	0.2

Table V. *Protective action of dialysed and undialysed liver extracts against the oxidation of vitamin C*

*Reaction mixture.* 10 ml. glycine buffer *M*/5 (pH 8.9); Cu as CuSO<sub>4</sub>, 5H<sub>2</sub>O (0.062 mg.); 5 ml. 0.100% vitamin C; 5 ml. liver extract; total volume, 40 ml. Temperature, 35  $\pm$  0.1°.

Time in min. ...	mg. vitamin C in total volume of reaction mixture						
	Undialysed liver extract			Dialysed liver extract			
	0	30	60	0	30	60	
1. Vitamin C	5.0	4.3	4.0	5.0	4.4	3.8	
2. Vitamin C + Cu	5.0	2.2	1.6	5.0	2.1	1.4	
3. Vitamin C + liver extract	5.0	4.8	4.6	5.0	4.4	4.0	
4. Vitamin C + Cu + liver extract	5.0	4.0	3.4	5.0	1.7	0.8	

is due to the presence of certain substances in the undialysed extract, which protect the vitamin against catalytic oxidation, so that the activity of the phosphatase is unaffected in the presence of vitamin C and copper. When these substances are removed by dialysis, the extract loses its protective properties, and the phosphatase is inhibited by the vitamin C-Cu complex because under such conditions the vitamin is oxidized. Further, the results show that, as in the case of kidney extracts, the dialysed extracts of liver contain some factors which accelerate the oxidation of vitamin C by copper.

*The influence of glutathione (GSH) on the activities of the alkaline and acid phosphatases of kidney and liver in the presence and absence of vitamin C-Cu complex*

The results of experiments on the effect of glutathione on the alkaline and acid phosphatases of kidney and liver in the presence and absence of the vitamin C-Cu complex are presented in Table VI.

The results show that glutathione (GSH) protects the acid phosphatase of kidney from inhibition by the vitamin C-Cu complex, while the inhibition of the activity of the alkaline phosphatases of kidney and liver is not greatly

Table VI. *Effect of glutathione on the activities of the alkaline and acid phosphatases of kidney and liver in the presence and absence of vitamin C-Cu complex.*

*Reaction mixture.* (Kidney phosphatases): 20 ml. glycine buffer *M*/5 (*pH* 8.9), for alkaline phosphatase and 20 ml. acetate buffer *M*/5 (*pH* 5.0) for acid phosphatase; 10 ml. 2% Na- $\beta$ -glycerophosphate; 4 ml.  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.1 *M* (only for alkaline phosphatase); 5 ml. enzyme (dialysed extract); vitamin C, 5 mg.; glutathione, 5 mg.; Cu as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.062 mg.); total volume, 50 ml.

(Liver phosphatase): 10 ml. glycine buffer *M*/5 (*pH* 8.9); 10 ml. 2% Na- $\beta$ -glycerophosphate; 4 ml. 0.1 *M*  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ; 5 ml. enzyme (dialysed extract); vitamin C, 5 mg.; Cu as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.062 mg.); glutathione, 5 mg.; total volume, 40 ml. Temperature,  $35 \pm 0.1^\circ$ .

## Activity in mg. P in 10 ml. reaction mixture

	Kidney alkaline phosphatase		Kidney acid phosphatase 12 hr.	Liver alkaline phosphatase	
	0.5 hr.	1 hr.		2 hr.	4 hr.
1. Purified phosphatase	0.400	0.640	0.200	0.457	0.800
2. Purified phosphatase + vitamin C	0.400	0.640	0.200	0.450	0.789
3. Purified phosphatase + vitamin C + Cu	0.310	0.490	0.040	0.333	0.580
4. Purified phosphatase + vitamin C + Cu + glutathione (GSH)	0.310	0.500	0.200	0.350	0.640
5. Purified phosphatase + glutathione (GSH)	0.400	0.571	0.182	0.444	0.762

## Oxidation of vitamin C

## Vitamin C in mg. in the total volume of reaction mixture

	Kidney alkaline phosphatase			Kidney acid phosphatase			Liver alkaline phosphatase		
	0	30	60	0	30	60	0	30	60
2. Purified phosphatase + vitamin C	5.0	3.6	3.0	5.0	4.8	4.8	5.0	4.5	4.1
3. Purified phosphatase + vitamin C + Cu	5.0	1.4	0.4	5.0	2.2	1.3	5.0	2.2	1.4
4. Purified phosphatase + vitamin C + Cu + glutathione	5.0	3.0	1.4	5.0	4.9	4.9	5.0	4.4	2.9

influenced in its presence. This difference in behaviour of glutathione is perhaps due to the fact that the vitamin is protected completely from oxidation at *pH* 5.0, which is the optimum for the acid phosphatase, while at *pH* 8.9, which is the optimum for the alkaline phosphatase, it has very little protective action. It is found that glutathione itself has a slight inhibiting action on the activity of the phosphatase.

## III. BRAIN PHOSPHATASE

*The influence of vitamin C on the activity of brain phosphatase*

*Preparation of extract.* *Extract No. 1.* 20 g. acetone-treated brain powder were extracted with 100 ml. water saturated with toluene for 48 hr. at room temperature and filtered.

*Extract No. 2.* Purified by isoelectric precipitation of the inert material at *pH* 4.8. The extract was prepared by adding an equal volume of *M*/5 acetate buffer *pH* 4.8 to the aqueous extract, and centrifuging to remove the suspended impurities.

*Extract No. 3.* Prepared by dialysing the crude aqueous extract of acetone-treated brain.

Table VII. *Influence of vitamin C on the phosphatase activities of dialysed and undialysed extracts of brain*

*Reaction mixture.* The composition was the same as that of the mixture used for kidney and liver phosphatases.

	Activity in mg. P in 10 ml. reaction mixture after 4 hr. hydrolysis	
	Undialysed extract	Dialysed extract
1. Phosphatase	0.158	0.110
2. Phosphatase + vitamin C	0.158	0.110
3. Phosphatase + vitamin C + Cu	0.090	0.060
4. Phosphatase + Cu	0.158	0.110

Oxidation of vitamin C

mg. vitamin C in the total volume of reaction mixture

Time in min.	...	Undialysed extract			Dialysed extract		
		0	30	60	0	30	60
2. Phosphatase + vitamin C		5.0	4.2	3.8	5.0	4.1	3.8
3. Phosphatase + vitamin C + Cu		5.0	2.6	1.6	5.0	2.5	1.4

The results show that, unlike kidney and liver phosphatases, the alkaline phosphatase of brain is inhibited by vitamin C-Cu complex irrespective of the purity of the enzyme. This is probably due to the absence of protective substances from the aqueous extracts of acetone-treated brain powder.

The results of the experiments on the protective action of brain extracts against the oxidation of the vitamin are presented in Table VIII.

Table VIII. *Influence of brain extracts on the oxidation of vitamin C*

*Reaction mixture.* 10 ml. glycine buffer *M*/5 (pH 8.9); 5 ml. 0.100% vitamin C; Cu as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.062 mg.); total volume, 40 ml.

	Time in min.	...	mg. of vitamin C in the total volume of reaction mixture							
			Undialysed extract				Dialysed extract			
			0	15	30	90	0	15	30	60
1. Vitamin C			5.0	4.8	4.6	3.8	5.0	4.6	4.3	4.0
2. Vitamin C + brain extract			5.0	4.8	4.8	4.4	5.0	4.6	4.3	4.0
3. Vitamin C + Cu + brain extract			5.0	3.2	2.6	0.3	5.0	2.8	2.2	1.1
4. Vitamin C + Cu			5.0	3.2	2.6	0.5	5.0	3.0	2.6	1.5

The results show that the protective action of the undialysed extracts of brain is very little compared with those of kidney and liver. The phosphatase is therefore inhibited by the vitamin C-Cu complex, because the vitamin is oxidized by copper under such conditions. Further, just as in the case of dialysed extracts of kidney and liver, brain extracts also accelerate the catalytic oxidations of vitamin C by copper. Experiments on the influence of the vitamin C-Cu complex on the activity of brain acid phosphatase have shown that, like the alkaline phosphatase, the acid phosphatase also is inhibited by the complex.

*The influence of glutathione on the vitamin C-Cu inhibition of the activities of the alkaline and acid phosphatases of brain*

The results of experiments on the influence of glutathione on the vitamin C-Cu inhibition of the activities of the alkaline and acid phosphatases of brain are presented in Table IX.

Table IX. *Effect of glutathione on the vitamin C-Cu inhibition of brain phosphatase*

*Reaction mixture.* 10 ml. glycine buffer *M*/5 (pH 8.9); 10 ml. 2% Na- $\beta$ -glycerophosphate; 4 ml. 0.1 *M* MgCl<sub>2</sub>, 6H<sub>2</sub>O; Cu as CuSO<sub>4</sub>, 5H<sub>2</sub>O (0.062 mg.); vitamin C, 5 mg.; glutathione, 5 mg.; total volume, 41 ml.

	Activity in mg. P in 10 ml. reaction mixture after 4 hr. hydrolysis	
	Alkaline phosphate	Acid phosphatase
1. Phosphatase	0.148	0.284
2. Phosphatase + vitamin C + Cu	0.121	0.172
3. Phosphatase + vitamin C + Cu + glutathione (GSH)	0.121	0.284
4. Phosphatase + glutathione (GSH)	0.138	0.284

Oxidation of vitamin C

mg. vitamin C in the total volume of reaction mixture

	Alkaline phosphatase					Acid phosphatase			
	Time in min. ...	0	15	30	60	0	30	60	120
2. Phosphatase + vitamin C + Cu		5.0	3.2	2.3	1.0	5.0	3.4	2.7	1.8
3. Phosphatase + vitamin C + Cu + glutathione (GSH)		5.0	4.4	4.2	3.3	5.0	4.8	4.7	4.7

The results show that glutathione (GSH) entirely prevents both the oxidation of vitamin C at pH 5.0 and the inhibition of the activity of the acid phosphatase by the vitamin C-Cu complex. The inhibition of the alkaline phosphatase activity is, however, not influenced by glutathione.

*The influence of cysteine, cystine, cyanide and sodium hydrosulphite on the inhibiting action of vitamin C-Cu complex*

It is well known that the catalytic influence of Cu on the oxidation of vitamin C is entirely or partly removed by glutathione (GSH), cysteine, cystine and cyanide [Hopkins & Morgan, 1936; De Caro & Giani, 1934]. Glutathione, which prevents the oxidation of the vitamin by metal catalysts by virtue of its ability to form a complex with Cu, is found to annul the inhibiting action of the vitamin C-Cu complex on the activity of the phosphatase. It was thought that the inhibition of the activity of the phosphatase by vitamin C might also be annulled by other substances which prevent the oxidation of the vitamin in the presence of Cu. It was found that such compounds as cysteine, cystine and cyanide, when added to the reaction mixture, either diminished or entirely eliminated the inhibiting action of the vitamin C-Cu complex. Sodium hydrosulphite, which is a reducing agent, was also found to protect the phosphatase from the inhibiting action of the complex.

The results are presented in Table X.

Table X. *Influence of cysteine, cystine, cyanide and sodium hydrosulphite on the inhibiting action of vitamin C-Cu complex*

*Reaction mixture.* 20 ml. acetate buffer *M*/5 (*pH* 5.0); 10 ml. 2% Na- $\beta$ -glycerophosphate; 10 ml. enzyme (purified by isoelectric precipitation of the impurities at *pH* 4.8); vitamin C, 5 mg.; Cu as CuSO<sub>4</sub>·5H<sub>2</sub>O (0.062 mg.); total volume, 51 ml. Cysteine, 5 mg.; cystine, 5 mg.; NaCN, 0.001 *M*; Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, 0.004 *M*.

Preparation	Substance tested	Reaction mixture	Activity in mg. P in 10 ml. of reaction mixture after 4 hr. hydrolysis
Brain No. 2	Cysteine	Phosphatase	0.231
		Phosphatase + vitamin C + Cu	0.122
		Phosphatase + vitamin C + Cu + cysteine	0.228
		Phosphatase + cysteine	0.220
Brain No. 3	Sodium cyanide	Phosphatase	0.308
		Phosphatase + vitamin C + Cu	0.160
		Phosphatase + vitamin C + Cu + NaCN	0.308
		Phosphatase + NaCN	0.297
Brain No. 4	Cystine	Phosphatase	0.320
		Phosphatase + vitamin C + Cu	0.173
		Phosphatase + vitamin C + Cu + cystine	0.280
		Phosphatase + cystine	0.320
Brain No. 4	Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	Phosphatase	0.320
		Phosphatase + vitamin C + Cu	0.173
		Phosphatase + vitamin C + Cu + Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	0.292
		Phosphatase + Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	0.310

## DISCUSSION

The results obtained in the present investigation on the interaction of vitamin C and tissue phosphatases (kidney, liver and brain) have confirmed the view-point developed by the author previously [Giri, 1938, 1 and 2], that the activity of the phosphatase is related to the oxidation of the vitamin. The phosphatases of all tissues are inhibited by the vitamin C-Cu complex, while the vitamin or copper alone has no effect on the activity. Further, it was found that whereas the phosphatase present in purified extracts of tissues is inhibited by the vitamin C-Cu complex, this is not true of the phosphatases present in the crude aqueous extracts under similar experimental conditions. These results are of particular interest since they suggest that the influence of vitamin C on the activity of tissue phosphatases is associated with certain factors present in the tissues, which protect the vitamin against oxidation.

These experiments have an interesting physiological significance. It seems likely that normal tissues always contain sufficient protecting substances to enable the enzyme to act at its full capacity within the cell in the presence of vitamin C and Cu, and that when these substances are removed or their effective concentration decreased by some means, the activity of the enzyme is adversely affected by the vitamin in association with Cu. Thus the interaction of vitamin C, Cu and protective substances like glutathione, which occur together in all tissues, may play an important role in the regulation of the activity of tissue phosphatases.

## SUMMARY

1. The activities of the alkaline and acid phosphatases of kidney, liver and brain are inhibited by the vitamin C-Cu complex. Vitamin C or Cu alone has no effect on their activity.

2. The phosphatase activity of crude aqueous extracts of tissues, with the exception of that of brain, is not influenced by the vitamin C-Cu complex, while the activity of extracts purified by dialysis is inhibited by the complex. This difference in the behaviour of the extracts was found to be due to the presence of factors in crude extracts which protect the vitamin against oxidation.

3. The inhibition by vitamin C-Cu complex was entirely or partly annulled by glutathione, cysteine, cystine and reducing agents like NaCN and  $\text{Na}_2\text{S}_2\text{O}_4$ .

4. It is suggested that vitamin C, Cu and protective substances like glutathione, which occur together in all tissues, play an important role in the regulation of the activity of tissue phosphatases.

I wish to express my thanks to the Indian Research Fund Association and to Dr W. R. Aykroyd, Director, Nutrition Research Laboratories, Coonoor, for providing me with facilities for work in the Laboratories.

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# XXXIX. THE CONVERSION OF CAROTENE TO VITAMIN A<sub>2</sub> BY SOME FRESH-WATER FISHES

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FRESHWATER fish contain a substance akin to, but not identical with, vitamin A, which on account of its distribution, chemical, physical and biological properties may properly be designated vitamin A<sub>2</sub>. It is differentiated from vitamin A by means of its ultraviolet absorption spectrum and by the antimony trichloride colour test:

	Vitamin A	Vitamin A <sub>2</sub>
U.V.	$\lambda_{\max}$ 328 m $\mu$	350 m $\mu$ (284 m $\mu$ ?)
Colour test	$\lambda_{\max}$ 617 m $\mu$ [583 m $\mu$ ] inflexion	693 m $\mu$ [645 m $\mu$ ] inflexion

The available evidence suggests strongly that some of the functions of vitamin A in salt-water fish are fulfilled in freshwater fish by vitamin A<sub>2</sub>. The only circumstances under which vitamin A<sub>2</sub> has been found to occur in mammals or birds are those which indicate the presence of vitamin A<sub>2</sub> in the diet (e.g. when freshwater fish are eaten freely) and there is no evidence of the natural synthesis of vitamin A<sub>2</sub> from provitamins except in fishes.

It is accepted that for the mammal  $\alpha$ - and  $\beta$ -carotenes (and a few related substances) act as provitamins A, since the administration of such materials results in the relief of symptoms of vitamin A deficiency. If supplied in relative abundance they markedly increase the vitamin A content of the liver.

The origin of the large amounts of vitamin A found in some fishes has never been determined. It is true that phytoplankton contains carotene, but the transformation of carotene into vitamin A has not been directly demonstrated for fishes, nor is there satisfactory evidence that the amount of carotenoid ingested is sufficient to account for the stored vitamin. The constitution of vitamin A<sub>2</sub> remains somewhat speculative and little is known concerning possible or actual precursor substances.

The object of the present series of experiments is to ascertain something of the effect of carotene added to the food of freshwater fish. The literature affords little guidance in the planning of such work. Search was made for a readily accessible freshwater species having the following characteristics:

- (1) the fish should be small enough to allow several to live in an aquarium;
- (2) the normal vitamin reserves should not be very high;
- (3) both vitamin A and vitamin A<sub>2</sub> should be present in easily detectable amounts in the healthy fish on a normal diet.

The most suitable species for a first experiment seemed to be the perch (*Perca fluviatilis*). A number of live fish were secured from Lake Windermere<sup>1</sup> and transported to Liverpool by road in suitable fish tanks. They were transferred on arrival to large glass tanks shaded from direct light and aerated by

<sup>1</sup> The co-operation of the Staff of the Freshwater Biological Association at Wray Castle is gratefully acknowledged.

circulating tap water carrying with it air bubbles. The inlet water was maintained for a time at a slightly higher temperature than that of the room, but after the fish had become habituated to their new surroundings the precaution was found to be unnecessary. It was realized almost at once that too many fish had been placed in the aquaria and a number were removed with a net and pithed. These were then dissected and the different organs tested for carotenoids, vitamin A and vitamin A<sub>2</sub>. A few fish died in the first few days and all refused food. This experience is unfortunately common with fish unused to captivity. Search was made for a suitable live food and it was found that the water shrimp *Gammarus neglectus*, which was fortunately available in quantity (River Weaver, Cheshire) proved tempting. After fasting for nearly a fortnight all the perch took the shrimp with avidity and a plentiful supply for about 14 days sufficed to restore a normal appetite.

There were, however, two objections to continuing to feed the fish on water shrimps. In the first place, much work is needed to obtain a sufficient, regular supply of live shrimps and the task of breeding them in the laboratory threatened to be beyond us. Secondly, it was not possible to regard the diet as satisfactory for the experiments which had been planned.

Thus, 28 g. of dead shrimps were completely decomposed by means of alcoholic potash and the non-saponifiable fraction was extracted. It contained a carotenoid pigment ( $\lambda_{\max}$  484, 453, 428 ~ 407 m $\mu$ , ~ denoting inflexion) and gave maxima at 617 m $\mu$  and 583 m $\mu$  with the SbCl<sub>3</sub> reagent.

$E_{1\text{ cm}}^{1\%}$	620 m $\mu$	0.037
	583 „	0.029
calculated on the weight of shrimp		

Similar tests on a further 23 g. of shrimp gave:

$E_{1\text{ cm}}^{1\%}$	620 m $\mu$	0.048
	583 „	0.036

and direct absorption spectra confirmed the presence of carotenoid with additional maxima at 344, 316 ~ 286 and ~ 276 m $\mu$ .

These results are certainly an indication that the water shrimp contains small quantities of material giving tests very similar to those of vitamin A. It would be necessary to carry out a chromatographic analysis of the non-saponifiable fraction in order to be certain which carotenoids are present and to give rigorous proof that the colour test was due to vitamin A rather than to carotenoids. In any case, the shrimp was a poor carrier for adding carotene to the diet. Moreover, it probably contains astacene which may be a vitamin A precursor in some fish.

Various foods were then tried. The digestive glands of *Mytilus* proved on the whole unsatisfactory, minced lean beef was only sparingly eaten, but after some initial reluctance all the fish took blow-fly larvae with great readiness.

At the end of a preliminary period of six weeks the perch were accustomed to the aquaria and could be fed without difficulty. The diet consisted of larvae smeared occasionally with herring-body oil (of negligible vitamin A content). On some days finely chopped raw lean beef was provided.

The next problem concerned the administration of carotene. The provision of an exact amount of carotene in the daily diet of the perch seemed impossible. The object of the experiment was to test the effect of a diet substantially enriched with carotene, and it was necessary to become reconciled to a considerable wastage of carotene in order to be sure that a fair quantity would actually be ingested.



The material used was a sample of leaf carotene containing both  $\alpha$ - and  $\beta$ -carotenes but little or no "xanthophyll".

Finely ground solid carotene was placed in a glass dish and clean larvae were picked out with a pair of forceps and dropped into the dish. By gently rubbing the live larvae in the powdered carotene they were easily coated with a minute staining layer which adhered fairly strongly. The coated larvae were fed to the perch two or three at a time and were taken at once. Feeding was on each occasion discontinued as soon as the fish lost interest. It was at first thought that one meal every 48 hours would be best but experience showed that the fish were ready to eat once each day. A little extra food provided at noon on Saturdays had been eaten before Monday morning. Care was taken to remove food debris from the aquaria and to ensure constant aeration. A group of fish maintained in a separate tank received a diet as nearly as possible identical with that supplied to the experimental group, except that the controls received no added carotene.

After a period of two months the controls were removed by means of a net and at once killed by pithing. They were then dissected and the various portions assembled for analysis. Each sample was refluxed with alcoholic potash and the non-saponifiable fractions were extracted with ether. The ether extracts were dehydrated by treatment with pure alcohol and nitrogen on a water bath. The non-saponifiable fractions were then subjected to the  $\text{SbCl}_3$  colour test and the vitamin concentrations estimated by spectroscopic examination.

### Experimental data

Perch from Lake Windermere—not fed in captivity

(a)		(b)	
8 fish: 4♂, 4♀		3 fish	
Average wt. 38 g., average length 14.9 cm.		1.253 g., average length 0.42 g.	
Livers: 3.274 g., average 0.42 g.			
$E_{1\text{ cm}}^{1\%}$ { 693 m $\mu$ 0.04 640 " 0.075 Feeble maxima at 595 and 560 m $\mu$		$E_{1\text{ cm}}^{1\%}$ { 693 m $\mu$ 0.018 640 " 0.044 605 " 0.053 560 " 0.043	
Viscera (comprising hearts, spleens, gonads, mesenteries): 20.3 g., average 2.2 g.		Eyes	Feeble blue colour
$E_{1\text{ cm}}^{1\%}$ { 695 m $\mu$ 0.01 636 " 0.011 556 " 0.09		Pyloric caeca	"
		Intestines	"
		Mesenteric fat	"
Alimentary tracts: 6.98 g., average 0.87 g.		Gonads	No blue colour
$E_{1\text{ cm}}^{1\%}$ { 693 m $\mu$ 0.008 600 " Transient 620 "		Hearts	"
		Spleens	"
		Stomachs	"

Eyes and bodies both gave feeble colour tests

Controls: 6 fish fed in captivity, no added carotene

Livers: 3.71 g., average 0.62 g.

$E_{1\text{ cm.}}^{1\%}$ { 693 m $\mu$ 0.064 620 " 0.040		
Mesenteries: 3.58 g., average 0.6 g.		
$E_{1\text{ cm.}}^{1\%}$ { 695 m $\mu$ 0.0116 655 " 0.0124 620 " 0.0124 580 " 0.0085		
Eyes	Very faint blue colour	
Pyloric caeca	No blue colour	
Hearts	"	
Spleens	"	
Stomachs	"	
Intestines	"	
Gonads	"	

## Perch on a diet enriched with carotene

3♀ immature, 3♀ adult, 2♂ adult

Average wt. 36.3 g., average length 15.8 cm.

Livers: 4.28 g., average 0.61

$E_{1\text{ cm.}}^{1\%}$	693 mμ	0.206	
	620 "	0.086 only 693 mμ band seen	
$E_{1\text{ cm.}}^{1\%}$	645 mμ	0.0086	
	605 "	0.0096	
	592 "	0.0086	

Pyloric caeca: 2.83 g.

Faint blue colour

Intestines: 3.86 g.

Faint blue (greenish blue)

Gonads: 17.43 g.

Faint blue

Eyes: 5.78 g.

"

Stomachs: 2.32 g.

No blue colour

Hearts: 0.47 g.

"

Spleens: 0.28 g.

"

It is clear that vitamin A and vitamin A<sub>2</sub> occur preferentially in the livers. Although in an absolute sense the concentrations are low, the amount of vitamin A<sub>2</sub> is very definitely increased as a result of the administration of dietary carotene.

	$E_{1\text{ cm.}}^{1\%}$ 693 mμ
Windermere fish feeding naturally	0.04
Controls	0.018
Carotene-fed perch	0.064
	0.206

The control fish had, as it turned out, rather larger reserves than those fresh from the open lake. There can be little doubt that the threefold increase in vitamin A<sub>2</sub> and the twofold increase in vitamin A are consequent upon the administration of carotene.

A few months later, perch from a different locality became available after feeding naturally until July, 1938. They had thus had the benefit of the photo-synthetic activity in spring and early summer and it would be reasonable to expect that they had obtained full access to the natural provitamins A.

## Perch from Aberdeen

20 fish, average wt. 225 g.

Livers 73 g. (average 3.65 g.). After refluxing with alcoholic potash and extraction with ether 0.33 g. of non-saponifiable matter was isolated:

		On non-sap.	On liver	
$E_{1\text{ cm.}}^{1\%}$	693 mμ	132	0.6	Colour test
	617 "	46	0.21	
	351 "	36.4	0.165	
				u.v. absorption

It will be seen that the ratio  $E_{1\text{ cm.}}^{1\%}$  693/617 mμ is 2.87, showing that vitamin A<sub>2</sub> is present in much larger amount than vitamin A. The absolute quantity, c. 120 p.p.m., is not high but is nevertheless three times as great as that shown by the carotene-fed perch and some nine times as great as that shown by the controls. The ultraviolet absorption spectrum shows, in addition to the 351 mμ maximum characteristic of vitamin A<sub>2</sub>, a second band at 284 mμ which may be due to a third absorbing entity in the non-saponifiable fraction.

It will be noticed that these perch were on the average much larger (six to seven times by weight) than those obtained from Windermere. The livers from the

present batch of perch averaged 3.65 g. as against 0.6 g. for the experimental fish. The much higher storage reserves of vitamin A<sub>2</sub> in the larger and (probably) older fish is in good accord with experience obtained with the vitamin A contents of cod and halibut livers. It seems likely that in any year the fish tends to show a positive balance in its vitamin A or A<sub>2</sub> economy resulting in greater storage with age.

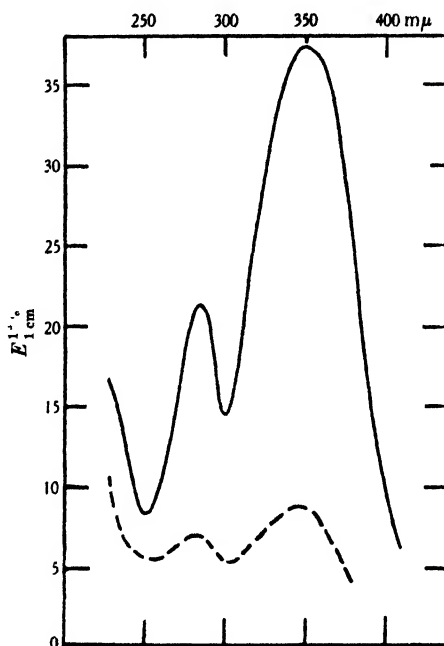


Fig. 1. Ultraviolet absorption spectrum of non-saponifiable fractions from (a) perch liver oil —; (b) perch intestinal oil ---.

*Stomachs.* 30 g. of tissue yielded 0.1318 g. (4393 p.p.m.) of non-saponifiable matter. A 13.2% solution of the "non-sap." diluted with 10 vol. of the  $\text{SbCl}_3$  reagent gave a blue solution by means of which it was just possible to recognize the 693 mμ maximum but not the 617 mμ band. The vitamin content of the stomachs must therefore have been negligibly small.

*Eyes.* The whole eyes from 20 perch weighed 47 g. Prolonged treatment with hot alcoholic potash followed by the usual extraction process gave 0.9563 g. (20,350 p.p.m.) of non-saponifiable matter. This material gave a good blue colour with the  $\text{SbCl}_3$  reagent:

$E_{1\text{ cm.}}^{1\%}$	693 mμ	Not measurable
	642 "	0.315
	600 "	0.294
	565 "	0.28

Three absorption bands were seen clearly. The eyes evidently contain small quantities of both vitamin A and vitamin A<sub>2</sub> although the rest of the non-saponifiable fraction exerts marked inhibition in the colour test. The eyes probably yielded 2 p.p.m. vitamin A and 3–4 p.p.m. vitamin A<sub>2</sub>.

*Intestines.* 60 g. yielded 0.353 (5900 p.p.m.) of non-saponifiable matter:

On non-sap.		
$E_{1\text{ cm.}}^{1\%}$	693 mμ	23
	617 "	9.3

The ratio  $E_{1\text{ cm}}^{1\%}$  693 m $\mu$ /617 m $\mu$  is 2.47, i.e. vitamin A<sub>2</sub> does not preponderate quite so much as in the livers. The ultraviolet absorption shows two maxima, one near 350 m $\mu$  and the other near 285 m $\mu$ . The relative intensities of these two maxima in the intestinal non-saponifiable fraction are so strikingly different from the relative intensities shown by the liver "non-sap." that little doubt can remain that the two maxima belong to distinct compounds.

*Mesenteric fat.* A good deal of mesentery could be detached from the ileum of each fish. 68 g. yielded 0.098 g. of "non-sap.":

On non-sap		
$E_{1\text{ cm}}^{1\%}$	{ 693 m $\mu$	22.5
	{ 617 "	10.7

corresponding to c. 2.5 p.p.m. vitamin A and c. 6.5 p.p.m. vitamin A<sub>2</sub>.

The fact that carotene can act as provitamin A and provitamin A<sub>2</sub> for the perch seems established from this work, but the failure to raise the storage levels to that observed in larger fish feeding naturally is at first sight disturbing. There is little reason to suppose that carotene is the sole precursor substance. In fact the amounts of carotene available in the natural diet are rather surprisingly small.

Thus a sample of 53.8 g. of zooplankton supplied from Windermere (by courtesy of the Freshwater Biological Research Station, Wray Castle) after saponification yielded a fraction unmistakably containing carotene but so contaminated with other absorbing substances as to preclude a quantitative assay. The acids recovered from the soaps were deeply coloured and gave numerous absorption bands ( $\lambda_{\text{max}}$  271, 283, 301, 317, 330, 349, 377, 405, 450, 482 m $\mu$ ) indicating the presence of conjugated polyenes. The quantities available did not permit of fractionation.

A further experiment was made on dace fed on blow-fly larvae with and without added carotene:

#### *Experiments on dace*

Controls		Diet enriched with carotene	
5 fish		4 fish	
Livers: 8.78 g.		7.4 g.	
$E_{1\text{ cm}}^{1\%}$ { 693 m $\mu$	0.19	$E_{1\text{ cm}}^{1\%}$ { 693 m $\mu$	0.73 Colour
	{ 620 " 0.19		{ 620 " 0.65 test
			{ 345 " 0.28 c.v. test
Vitamin A c. 38 p.p.m.		Vitamin A c. 130 p.p.m.	
Vitamin A <sub>2</sub> c. 38 p.p.m.		Vitamin A <sub>2</sub> c. 146 p.p.m.	
Stomachs: 1.45 g.		1.5 g.	
$E_{1\text{ cm}}^{1\%}$ { 693 m $\mu$	0.011	$E_{1\text{ cm}}^{1\%}$ { 693 m $\mu$	0.35
	{ 620 " 0.027		{ 620 " 0.70
			{ 583 " 0.38
Vitamin A 5.4 p.p.m.		Vitamin A 140 p.p.m.	
Vitamin A <sub>2</sub> 2.2 p.p.m.		Vitamin A <sub>2</sub> 70 p.p.m.	
Intestines: 1.25 g.		1.14 g.	
$E_{1\text{ cm}}^{1\%}$ 620 m $\mu$	0.027	$E_{1\text{ cm}}^{1\%}$ { 693 m $\mu$	0.07
			{ 620 " 0.225
Vitamin A 5.4 p.p.m.		Vitamin A 45 p.p.m.	
		Vitamin A <sub>2</sub> 14 p.p.m.	
Rest of viscera: 8.1 g.		5.3 g.	
Vitamin A 2.4 p.p.m.		Vitamin A 4 p.p.m.	
Vitamin A <sub>2</sub> 2.0 p.p.m.		Vitamin A <sub>2</sub> 3.2 p.p.m.	
Gonads: 27.4 g.		40.8 g.	
Vitamin A 0.84 p.p.m.		Vitamin A 1 p.p.m.	
Vitamin A <sub>2</sub> 0.74 p.p.m.		Vitamin A <sub>2</sub> 1 p.p.m.	
Eyes: Trace of vitamins A and A <sub>2</sub>		Trace of vitamins A and A <sub>2</sub>	

The work on dace shows a substantial increase in the amounts of both vitamins A and A<sub>2</sub> as a result of adding carotene to the diet. The marked increase in the alimentary tract is striking and suggests that the possibility of conversion of carotene in the stomach and intestines requires further investigation.

An attempt was made to extend the experiments to chub. The controls (2) on the carotene-free diet were unsatisfactory, as they could not be induced to eat freely. They may have been injured before arrival, but when it was seen that they were not doing well they were removed by means of a net, pithed and examined. Their vitamin reserves were low. The fish receiving a diet enriched with carotene compared badly with later specimens feeding naturally:

	Chub fed on blow-fly larvae plus carotene			Freshly caught chub (Mar. 1938)		
	Wt. g.	P.p.m. vitamin		Wt. g.	P.p.m. vitamin	
		A	A <sub>2</sub>		A	A <sub>2</sub>
Livers	8.4	39	33	6.9	120	50
Intestines	3	4.3	1.5	1.33	75	43
Stomachs	4.2	3.2	1.6	1.31	110	6
Rest of viscera	4.1	0.2	1.9	4.73	94	42
Eyes		Traces			Traces	
Gonads		Traces			Traces	

The chub did not take kindly to captivity, and this experiment is unsatisfactory. The striking difference between the contents of the alimentary tracts in respect of vitamins A and A<sub>2</sub> is worth emphasis.

#### SUMMARY

Perch and dace in captivity appear to thrive for a considerable period on a diet of blow-fly larvae. When this diet is enriched with carotene for a few weeks the store of vitamins A and A<sub>2</sub> increases considerably. From this it is concluded that carotene acts as provitamin for the formation *in vivo* of both vitamins. Whatever the constitution of vitamin A<sub>2</sub> may be it cannot be very different from that of vitamin A.

We are indebted to Dr J. A. Lovern, Dr F. J. Daniel, Mr G. W. Simpkins and Mr F. Dawson for valuable assistance. Thanks are also due to the Medical Research Council for a grant which enabled this work to be carried out.

# XL. THE DISTRIBUTION OF VITAMINS A AND A<sub>2</sub>. II

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(Received 25 January 1939)

IN the present phase of research on the A-vitamins two facts merit special attention. One concerns the distribution of the vitamins in respect of the absolute amounts found at different sites in different species and the other the relative proportions of the two vitamins.

The first fact is that a great deal of vitamin occurs in the absorptive parts of the alimentary tracts of many, but not all, species of fish. The second is that vitamin A<sub>2</sub>, which is practically confined to fishes, shows in all species a distribution over the body which differs appreciably from that of vitamin A. Vitamin A<sub>2</sub> predominates over vitamin A only in fresh water fishes; it has, however, been detected in birds subsisting on fish from inland waters.

Earlier work [Edisbury *et al.* 1938] on the distribution of the two vitamins has been extended.

## EXPERIMENTAL

### *Sturgeon (Acipenser sturio)*

A large sturgeon 10 ft. 6 in. long was landed at Aberdeen in May, 1938. The viscera were obtained and extracted in the laboratory.

The pyloric caeca, which form a compact mass in the sturgeon, were missing and had probably been thrown away by the fish merchant. The stomach was not freed from adhering mesentery but showed a high oil content which could not be attributed to mesenteric fat.

Table I

	Wt. g.	Oil %	Wt. of oil g.	Vitamin in oil %	Wt. vitamin g.	Vitamin A p.p.m. in tissue
Liver	3941	37.2	1466	1	14.66	c. 3000
Intestines*	1541	1.9	29.3	10	2.93	1900
Stomach	233	25.7	60	0.01	0.006	26
Spleen	145	4.8	6.96	0.015	0.001	7
Mesentery	75	24.0	18	0.006	0.0011	14

\* Minus pyloric caeca.

Table II. *Spectroscopic tests on oils*

		Liver oil	Intestinal oil	Spleen oil	Stomach oil	Mesenteric oil
$E_{1\text{cm}}^{1\%}$	(693 mμ) Colour	11	40	Weak	Not detectable	Not detectable
	617 „ test	47	509	0.36	0.18 (600 mμ)	0.12 (600 mμ)
	583 „ SbCl <sub>3</sub>	26	272	0.38	0.23 (572 mμ)	0.16 (572 mμ)
	325 „ Direct absorption	17	157	—	—	—
		No inhibition of colour test		Marked inhibition of colour test with SbCl <sub>3</sub>		
Ratio $E_{1\text{cm}}^{1\%}$ 617/693 mμ		4.3	12.7			

This large sturgeon contained some 30 g. of vitamin A esters and perhaps 7 g. of vitamin A<sub>2</sub> esters in its liver. The complete intestines must have contained at least 6 g. of vitamin A esters and perhaps 0.5 g. of vitamin A<sub>2</sub> esters. These quantities are large for a single fish and support the possibility previously suggested that the A-vitamins possess functions not hitherto recognized.

Possibly vitamin A<sub>2</sub> is passed on to the liver from the intestines more readily than vitamin A, since the ratio A/A<sub>2</sub> is much higher in the intestines than in the liver. This is also true of salmon, trout and halibut [Edisbury *et al.* 1938]. The distribution of the two vitamins suggests that they may not exert identical functions at all sites.

*Lampern (Petromyzon fluviatilis)*

In order to illustrate the kind of contrast which has already been noticed between, for example, eels [Edisbury *et al.* 1937] and halibut [Edisbury *et al.* 1938], the sturgeon may be compared with the freshwater lamprey. Three sets of data were obtained and gave results in fair agreement.

Lamperns (Worcester), Nov. 1937. 6♂, 6♀. Total wt. 439 g. Average wt. 36.6 g.

						Bodies		
		Digestive glands	Fore-gut	Hind-gut	Gonads and excretory glands	Eyes	Head and thorax	Abdomen and tail
Wt. g.	...	6.45	1.4	1.28	35.4	1.0	109.5	284.5
$E_{1\text{cm}}^{1\%}$	693 mμ	Not seen	Not seen	0.16	0.13	—	0.01	0.007
	617 „	0.185	0.37	1.7	1.18	0.015	0.03	0.016
	583 „	0.12	0.2	0.9	0.6	—	0.023	0.012
	325 „	—	—	—	—	—	0.027	0.016
Approx. wt. of vitamin A, mg.		0.24	0.1	0.4	8.26	0.003	0.04	0.08
Vitamin A, p.p.m.		37	70	312	233	3	0.37	0.38

It is noteworthy that vitamin A predominates over vitamin A<sub>2</sub> and is very widely distributed in small amounts.

*Dogfish (Squalus acanthias)*

Some species are noteworthy for possessing abnormally low vitamin A reserves in the liver. Our experience of dogfish liver oil is that it commonly contains very little vitamin but it would be rash to generalize because we have examined one specimen (from Newfoundland) which was unusually rich. If a species tends to store little vitamin A in the liver, the present work makes it interesting to examine its intestines. We therefore retained a quantity of such material from dogfish.

558 g. of dogfish intestines were decomposed by means of alcoholic potash; 2.25 g. of non-saponifiable extract, obviously mainly sterol, were obtained. This material gave the following tests:

$$E_{1\text{cm}}^{1\%} \begin{cases} 693 \text{ m}\mu & 0.46 \\ 617 \text{ „} & 8.5 \\ 580 \text{ „} & 5.27 \end{cases}$$

The vitamin A content of the “non-sap.” is of the order 0.2 % so that the intestines contain:

c. 4000 p.p.m. of sterol,  
8 p.p.m. of vitamin A,  
0.3 p.p.m. of vitamin A<sub>2</sub>.

The contrast with halibut intestines containing up to 35,000 p.p.m. vitamin A is very striking and suggests a radical difference between the two species in respect of the mechanism of fat absorption (see Part III in this *Journal*, p. 330).

We have already noted [Edisbury *et al.* 1938] a marked decrease in the vitamin A content of the intestines of halibut as the absorptive function decreases in importance towards the vent. This has been confirmed. Thus the entire rectal caecum of one fish yielded 0.8% of oil containing 4.25% of vitamin, corresponding to 340 p.p.m., as against 33,000 p.p.m. for the absorptive parts of the gut.

The stomach of halibut is likewise poor in oil. The entire stomach of one fish yielded 0.5% of oil, 26.7% of which was non-saponifiable matter, mainly cholesterol. The vitamin A content was of the order 2 p.p.m. The stomach "oil", if dissolved in a suitable solvent to saturation at the boiling point, yields a well crystallized mixture of sterol and sterol esters on cooling. A halibut caught in Sept. 1938 was obtained with viscera in a good state of preservation. The stomach tissue was split up into various coats (see Part III in this *Journal*, p. 330) and the amount of non-saponifiable matter was determined:

	Wt. g.	Non-sap. p.p.m.
Layer 1 (inside)	214	3900
2	596	3200
3	263	4150
4	562	3070

The proportion of sterol did not exceed that usual in muscle cells, and the lining of the stomach contained little or no fat and practically no vitamin A.

The sterols from the various layers were recrystallized and some were obtained pure enough to warrant quantitative determinations of provitamin D content (probably 7-dehydrocholesterol). Cholesterol from cod liver oil, salmon liver oil and similar sources shows the ergosterol-7-dehydrocholesterol absorption bands at 293.5, 281.5 and 270  $\mu$ ,  $E_{1\text{ cm}}^{1\%}$  281.5  $\mu$  0.1 to 0.3 corresponding to some 0.03–0.1% of provitamin. The sterol from halibut stomach also shows these bands but at much higher intensity,  $E_{1\text{ cm}}^{1\%}$  281.5  $\mu$  6.2 and 4.5. No explanation of this has been found, but it is possible that the provitamin D is a product of the hydrogenation of cholesterol occurring in the tissue.

Halibut spleen from the same fish was also studied. It yielded c. 5300 p.p.m. of non-saponifiable matter, c. 380 p.p.m. of vitamin A and c. 35 p.p.m. of vitamin A<sub>2</sub>. Recrystallization of the non-saponifiable matter from methyl alcohol gave sterol in good yield. One further recrystallization gave a snow-white sample of cholesterol and an almost colourless mother liquor. Spectroscopic examination of both sterol and mother liquor revealed highly selective absorption of an unexpected type. The broad persistent absorption band with  $\lambda_{\text{max}}$  275  $\mu$  showed no sign of resolution into the narrow bands characteristic of ergosterol and dehydrocholesterol. The intensity of absorption,  $E_{1\text{ cm}}^{1\%}$  275  $\mu$  4.6, in the recrystallized sterol was by no means negligible and perhaps indicates that the cholesterol was contaminated with 1–2% of a substance not so far identifiable.

Similar experiments on bullock spleen and sheep spleen failed to show the presence of this absorbing impurity in mammalian spleen.

Bullock spleen:

Non-sap. 5200 p.p.m.

Carotene c. 1.5 p.p.m.

Recrystallized sterol showed three bands corresponding with those of ergosterol and 7-dehydrocholesterol (0.02% in the cholesterol).



## Sheep spleen:

Non-sap. 4800 p.p.m.

Carotene 0.26 p.p.m.

Recrystallized sterol contained c. 0.06 % of "dehydrocholesterol".

The amount of vitamin A in the bullock and sheep spleens was extremely minute.

*Vitamin A in sea-birds*

In view of the considerable, though variable, amounts of vitamin A occurring in the absorptive parts of the intestines of fishes, it seemed desirable to study its distribution in birds subsisting mainly on fish. The herbivorous mammal does not retain more than a few parts per million of vitamin A in its intestines. The following data must be regarded as preliminary to further work. The figures indicate, however, the presence of appreciable amounts of vitamin in the intestines.

*Herring gull (Larus argentatus)*

The liver (29 g.) yielded 0.172 g. of non-saponifiable matter, 23 % of which (0.04 g.) consisted of vitamin A corresponding to 1380 p.p.m. in the tissue. Vitamin A<sub>2</sub> could not be detected.

The intestines (43 g.) yielded 0.194 g. of non-saponifiable matter containing 2.9 % (0.0056 g.) of vitamin A or 130 p.p.m. Vitamin A<sub>2</sub> was again absent.

The heart and lungs both gave extremely low values (<1 p.p.m.) for vitamin A.

*Skua gull (Megalestris catarrhactes)*

The livers from 8 birds weighed 300 g. and yielded 2.2 g. of non-saponifiable extract containing 32 % (0.7 g.) of vitamin A. This corresponds to c. 2300 p.p.m.

The intestines (345 g.) contained 1.8 g. of non-saponifiable matter, 4 % of which was vitamin A. This is equal to 208 p.p.m. of vitamin.

The hearts, eyes and lungs all contained less than 1 p.p.m. vitamin A. No vitamin A<sub>2</sub> was detected in any part of the birds.

*Gannet (Sula bassana)*

One bird was dissected. The lungs were very large (74 g.) and gave 0.341 g. of non-saponifiable extract, 19 % (0.065 g.) of which was vitamin A. This corresponds to 870 p.p.m.

The liver (5.5 g.) was abnormally small and only contained a few p.p.m. of vitamin A.

The intestines (54 g.) yielded 0.231 g. of non-saponifiable matter mainly sterol and the vitamin A content did not exceed 10 p.p.m. The heart (30.5 g.) appeared to contain 0.7 p.p.m. vitamin A.

If these results are to be believed, the lungs take precedence over the liver and intestines as sites for vitamin A deposits in this species. The data are very surprising but we have found no valid reason for their rejection. A number of gannets will be studied as they become available.

## SUMMARY

A large sturgeon yielded 1.47 kg. of liver oil containing 1 % of vitamin A, whilst part of the intestines gave 29.3 g. of oil containing 10 % of vitamin. The liver contained c. 30 g. vitamin A esters and 7 g. vitamin A<sub>2</sub> esters. The intestines probably contained 6 g. and 0.5 g. of vitamin A and vitamin A<sub>2</sub> esters

respectively. The ratio vitamin A/A<sub>2</sub> was much higher in the intestines than in the liver.

Lamperns are not rich in vitamin A but small quantities occur at a number of sites. Vitamin A predominates over vitamin A<sub>2</sub>.

The dogfish, which yields a liver oil poor in vitamin A, also possesses extremely little in its intestines. Those parts of the alimentary tract of the halibut which take little part in the assimilation of fat are likewise poor in vitamin and it may be that the dogfish and some other species do not use vitamin A in absorption to the same extent as the halibut, cod and salmon.

A beginning has been made with the study of the distribution of provitamin D in the cholesterol obtained from different organs. The non-saponifiable fraction of spleen is noteworthy for the occurrence of small quantities of a substance with  $\lambda_{\max}$  275 m $\mu$ . This substance has not so far been detected in mammalian spleens.

Sea birds (herring gull and skua) have much more vitamin A in their intestines than such animals as the rabbit. The case of the gannet is surprising in that its lungs contain relatively large quantities of vitamin.

The work as a whole leads to the conclusion that vitamins A and A<sub>2</sub> probably do not replace one another with equal readiness in all functions. The enormous variations in the vitamin A content of fishes' intestines make it probable that the mechanism of assimilation is different when vitamin A is a major intestinal constituent from that obtaining when the vitamin is a trace constituent.

The authors wish to express their thanks to the Medical Research Council for a grant towards the expenses of this work and to Mr R. H. Creed for valuable technical assistance.

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## XLI. THE DISTRIBUTION OF VITAMINS A AND A<sub>2</sub>. III

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THE presence of large quantities of vitamin A in the intestines of certain fish was recently recorded [Lovern *et al.* 1937; Edisbury *et al.* 1938]. Its distribution in various parts of the alimentary tract was studied and, in the case of the halibut, the stomach was found to contain only traces of the vitamin whereas the "fatty" material extracted from the pyloric caeca, duodenum and ileum contained up to 70% of vitamin A esters.

The object of the present paper is to record the distribution of vitamin A and its congener vitamin A<sub>2</sub> in the different coats of the intestinal tube.

The problem has been approached in two ways:

- (a) Photomicrography of sections cut transversely.
- (b) Determination by spectroscopic methods of the vitamin content of the different layers of tissue after mechanical separation.

Histological work directed towards ascertaining the distribution of vitamin A demands a departure from normal technique because the solvents which might be used for clearing the sections are capable of dissolving out the vitamin, whilst the usual methods of fat-staining are unlikely to discriminate sharply between glycerides and vitamin A esters. We were therefore led to study the possibilities of ultraviolet photomicrography. Vitamin A absorbs radiations in the near ultraviolet region but transmits freely the longer wave-lengths of visible light. In principle, therefore, localized deposits of vitamin A should be detectable by the comparison of photographs taken by means of ultraviolet light and visible light respectively, using the same section and microscope setting for both experiments.

The ordinary microscope transmits ultraviolet rays in the region 340–400 m $\mu$  and although the peak of the vitamin A absorption (328 m $\mu$ ) falls outside this range, the long-wave side of the broad absorption band includes a sufficient part of the spectrum transmitted.

The special glass used in ultraviolet fluorescence cabinets (Wood's glass or Chance's ultraviolet glass) transmits practically no visible light but is fairly transparent towards near ultraviolet rays. If, after taking a photomicrograph in the usual way with visible light, a sheet of such glass is placed between the light source and the microscope, a second photograph may be taken by means of ultraviolet light. The exposure, of course, will need to be much longer.

A simple test of the method is easily made. If three small drops of oil are placed adjacent to one another on a cover slip as follows:

- Drop 1. Olive oil (no vitamin A),
- Drop 2. Cod liver oil (low vitamin A content),
- Drop 3. Halibut liver oil (high vitamin A content),

and photographed in the ways suggested above it is found that the ordinary negative shows a similar degree of blackening for the three drops, whereas the negative obtained with ultraviolet radiation exhibits a decreasing blackening in the order 1, 2, 3.

#### EXPERIMENTAL

Preliminary experiments were carried out on the intestines of a large halibut. The fish was dissected at Aberdeen and the intestines were sent to Liverpool, in the frozen state, in a large Dewar flask. The material arrived still frozen and the flask could not be emptied until the contents had been thawed. Sections were cut by hand and mounted in a large vertical enlarging lantern. Photographs taken by projection using screened and unscreened light gave promising results. It was realized, however, that the intestines were not too well preserved and that autolysis had occurred, particularly on the inside. Further, the sections were not clear enough, the magnification was inadequate and the ultraviolet pictures were not in good focus.

In order to cope with these difficulties it was arranged to carry out further work at sea on absolutely fresh material. The only method of clearing the sections which seemed satisfactory was that of MacConaill [1937-8], namely, immersing the freshly cut sections in a homogeneous mixture of glucose and golden syrup. It was found that the ordinary microscope could readily be adapted for use. It was employed horizontally, the mirror being dispensed with. A "pointolite" bulb provided sufficient ultraviolet radiation for the purpose. These preliminary details having been settled, fishing for halibut was undertaken in Shetland waters in May, 1938. Adverse weather conditions unfortunately limited both the catch and the experimental work but enough was done to demonstrate the need for fresh material and for fairly thick sections.

A little later the intestines of a very large halibut became available at Aberdeen in good condition and, as a result of previous experience, much more satisfactory photographs were obtained. The best results were obtained with hand-cut slices, 1-2 mm. thick before clearing, though with such sections it was not possible to obtain a clear picture of the detailed structure. Many pairs of photographs were, however, obtained and in the best of these it was evident that the mucosal coats were much more opaque to ultraviolet light than to visible light, whilst the muscle layers showed little sign of such selective absorption.

The technique is capable of considerable refinement but in the present work it was thought better to approach the problem from a different angle.

Attempts were made, therefore, to isolate the mucosal tissue from the underlying muscle and skin, in order to extract the oils and determine the respective vitamin A potencies.

It was clear from the earliest trials that the inside coats were richer than the outside ones, both in respect of fat and vitamin, but, owing to autolysis, the results lacked quantitative significance. For work of this kind it is desirable to use the very fresh viscera of a large halibut. The thick gut was opened longitudinally and the various coats were separated. Samples of such materials were (a) placed in alcohol and sent from Aberdeen to Liverpool for examination, and (b) extracted in bulk at Aberdeen. In general, lower values were obtained with the alcohol-treated samples, suggesting some destruction of vitamin.

*Terminology.* A thorough description of the anatomy and histology of the alimentary tract in the king salmon is given by Greene [1912]. We have already found that salmon, like halibut, contain considerable quantities of vitamin A in the intestines, and, as no comparable work on halibut was available to us, we have adopted the terminology used by Greene and supported by detailed drawings of histological sections.

The different coats in the intestines and pyloric caeca, from the lumen to the outer wall, are described as follows:

- I. *Mucosa*:
  - (a) Epithelium,
  - (b) *Tunica propria*,
  - (c) *Stratum compactum*,
  - (d) *Stratum granulosum*.
- II. *Muscularis*:
  - (a) *Circularis*,
  - (b) *Longitudinalis*.
- III. *Serosa*:
  - (a) *Serosa* proper,
  - (b) *Sub-serosa*.

The epithelial coat exhibits much folding but no villi and consists mainly of columnar epithelial cells, with mucous cells and wandering cells of the leucocyte type.

The *tunica propria* is made up of supporting connective tissue extending from the bases of the epithelial cells to the *stratum compactum*. It consists of white, fibrous tissue of the areolar type.

The *stratum compactum* is described by Greene as consisting of dense, non-fibrous connective tissue. The inner surface has projecting strands of the same type and the outer surface possesses two or three times as many of these processes, which form a network extending to the muscle coat. "Nowhere does there seem to be any direct opening or break through the thicker portion of the *stratum compactum* except where blood vessels penetrate this coat."

The *stratum granulosum* consists of granule cells enmeshed in the processes of the *stratum compactum*.

The muscle coats have been taken together in this work, as have those of the *serosa*, which consist of an outer "skin" and supporting connective tissue.

Two large halibut, one caught in May (A) and the other in September, 1938 (B), provided suitable material. In fishes generally the intestinal divisions are not so clearly differentiated as in mammals, but in this work a definite thinning of the intestinal wall was taken to mark the transition of duodenum to ileum.

After washing out food residues, a quantity of slimy matter, consisting of partly broken down mucosal epithelium, could be removed by very lightly scraping the inside of the gut. The wet weight of samples was taken before the addition of alcohol.

*Mucosal epithelium.* Sample 1a (18.76 g.) from the combined pyloric caeca and duodenum of fish A was covered with absolute alcohol and later ground in an agate mortar with sand and anhydrous sodium sulphate. The whole mass was thoroughly extracted with ether, giving 0.68 g. of fatty material (3.6%). Spectroscopic examination showed a vitamin A content of about 5.3%. The vitamin A content of the wet epithelium was therefore 0.19%, i.e. 1900 p.p.m. The ratio  $E_{1\text{ cm}}^{1\%} 617\text{ m}\mu/693\text{ m}\mu$ , representing the proportion of vitamin A to vitamin A<sub>2</sub>, was 10.8.

Sample 1b. The whole of the slime collected from the pyloric caeca, duodenum and ileum of fish A was subjected to direct fat extraction. The yield was 2.2% of an oil containing 12% of vitamin A, corresponding to 2680 p.p.m. The ratio  $E_{1\text{ cm}}^{1\%} 617\text{ m}\mu/693\text{ m}\mu$  (613/51) was about 12.

Sample 1c. Slime (40.04 g.) from the combined intestines of fish B, after grinding with sand and extraction with alcohol followed by ether, gave 3.01 g. of clear oil (7.5%). The spectroscopic characteristics of the oil:

$$E_{1\text{ cm}}^{1\%} \left\{ \begin{array}{lll} 693\text{ m}\mu & c. & 14 \\ 617 & \text{,,} & 170 \\ 583 & \text{,,} & 92 \\ 325 & \text{,,} & 54 \text{ u.v. absorption} \end{array} \right\} \text{ SbCl}_3 \text{ colour test}$$

were quite normal and corresponded to about 3.45% of vitamin A, or 2590 p.p.m. in the wet slime.

*Tunica propria.* After removal of the epithelial slime, this coat could be removed by harder scraping.

Sample 2a. This experiment was designed to secure a sample of oil in a state as near as possible to the natural one. The alcohol-preserved tissue from the pyloric caeca and duodenum of fish A was ground in an agate mortar with sharp sand and extracted in the cold with ether, a little alcohol being added occasionally to replace that lost in the successive extractions. 16.18 g. (wet weight) of tissue yielded 1.4 g. of oil, but the extraction was probably not quantitative. It contained 30 % of vitamin A.

Sample 2b. Direct extraction of the main bulk of the *tunica propria* from the pyloric caeca and duodenum yielded about 11 % of oil of similar potency:

$$E_{1\text{ cm}}^{1\%} \begin{cases} 693 \text{ m}\mu & 140 \\ 617 & \text{,,} & 1545 \\ 583 & \text{,,} & 825 \\ 325 & \text{,,} & 460; 445.5 \end{cases}$$

Taking these figures to correspond to some 30 % of vitamin A (c. 60 % vitamin A esters) the *tunica propria* contained some 33,000 p.p.m. The vitamin A<sub>2</sub> content is of the order 3000 p.p.m.

Sample 2c. The *tunica propria* scrapings (38 g.) from the ileum of fish A were preserved in alcohol and later, after grinding with sand and sodium sulphate, were exhaustively extracted with alcohol and ether, giving 11 % of pale, clear oil. The spectroscopic data:

$$E_{1\text{ cm}}^{1\%} \begin{cases} 693 \text{ m}\mu & 93.5 \\ 617 & \text{,,} & 1650 \\ 583 & \text{,,} & 900 \\ 325 & \text{,,} & 485 \end{cases}$$

correspond to 32.5 % of vitamin A, or 35,750 p.p.m. in the tissue.

Sample 2d. 37.4 g. of the *tunica propria* from the combined intestines of fish B gave 3.85 g. of oil (10.3 %) having the following characteristics:

$$E_{1\text{ cm}}^{1\%} \begin{cases} 693 \text{ m}\mu & 56 \\ 615 & \text{,,} & 900 \\ 583 & \text{,,} & 500 \\ 325 & \text{,,} & 275 \end{cases}$$

or 18 % vitamin A, corresponding to 18,500 p.p.m. in the tissue.

*Stratum compactum and stratum granulosum.* After scraping off the *tunica propria* it was found possible to detach the next coat with a sharp knife and to peel it off in long strips. This coat was taken to include both the *stratum compactum* and the enmeshed *stratum granulosum*.

Sample 3a. A small portion from the pyloric caeca and duodenum of fish A was preserved in alcohol and on extraction gave 3.28 % of oil, containing about 20 % of vitamin A:

$$E_{1\text{ cm}}^{1\%} \begin{cases} 693 \text{ m}\mu & 70 \\ 617 & \text{,,} & 1000 \\ 583 & \text{,,} & 530 \end{cases}$$

corresponding to some 6600 p.p.m. in the tissue.

Sample 3*b*. The bulk sample corresponding to the above yielded 3.1 % of oil containing about 29 % vitamin A:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 122 \\ 617 & \text{,,} & 1446 \\ 583 & \text{,,} & 735 \end{cases}$$

or about 9000 p.p.m. in the tissue.

Sample 3*c*. The coat from the ileum of fish A gave 1.8 % of oil containing 18 % of vitamin:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 56 \\ 617 & \text{,,} & 903 \\ 583 & \text{,,} & 490 \end{cases}$$

or some 3240 p.p.m.

Sample 3*d*. The coat from the combined intestines of fish B yielded 4.8 % of oil containing 28 % vitamin A:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 70 \\ 617 & \text{,,} & 1400 \\ 583 & \text{,,} & 717 \\ 325 & \text{,,} & 432 \end{cases}$$

or 13,440 p.p.m. in the tissue.

*Muscle coats.* After peeling off the previous coat it was possible to separate with a knife the muscle from the *serosa*. The muscle tissue is not effectively disintegrated by grinding with sand and, as the fat content is low, is best decomposed by refluxing with alcoholic potash.

Sample 4*a*. Using a small sample from the pyloric caeca and duodenum of fish A, preserved in alcohol, the non-saponifiable extract (0.23 %) was tested:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 16 \\ 617 & \text{,,} & 220 \\ 583 & \text{,,} & 135 \end{cases}$$

The vitamin content of 4.5 % corresponds to *c.* 103 p.p.m. in the tissue.

Sample 4*b*. The main bulk of the above material was worked up at once without treatment with alcohol. About 1.1 % of oil was obtained containing 2 % of vitamin A, or 220 p.p.m. of vitamin A in the tissue.

Sample 4*c*. The muscle coats from the ileum of fish A yielded 1 % of oil containing *c.* 0.5 % of vitamin A. The estimated amount lay between 50 and 70 p.p.m.

Sample 4*d* consisted of muscle from the combined intestines of fish B and was decomposed with alcoholic potash. The non-saponifiable matter (0.33 %) gave the following data:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 13.4 \\ 617 & \text{,,} & 283 \\ 583 & \text{,,} & 147 \\ 325 & \text{,,} & 91 \end{cases}$$

corresponding to 5.65 % or 187 p.p.m.

*Serosa.* This was always carefully freed from adhering mesentery.

Sample 5*a* (12.4 g.), from the pyloric caeca and duodenum of fish A preserved in alcohol, was treated with alcoholic potash and gave 0.3 % of non-saponifiable matter:

$$E_{1\text{ cm.}}^{1\%} \begin{cases} 693 \text{ m}\mu & 36 \\ 617 & \text{,,} & 470 \\ 583 & \text{,,} & 254 \end{cases}$$

containing *c.* 9.4 % vitamin A or 282 p.p.m. in the tissue.

Sample 5b. A bulk extraction of the above material gave 1.2% of oil containing 3.5% of vitamin, or 420 p.p.m.

Sample 5c, from the ileum of fish A, contained 1.55% of oil of which about 2% was vitamin A. This corresponds to 310 p.p.m.

Sample 5d, from the combined intestines of fish B, was saponified directly and yielded 0.48% of non-saponifiable matter containing 7% of vitamin A, or c. 339 p.p.m. in the tissue.

*Mesentery.* The mesenteric tissue adhering to the gut was also examined.

Sample 6a, composed of tissue from the entire intestines of fish A, was saponified after preservation in alcohol and gave 0.4% of non-saponifiable matter:

$$E_{1\text{ cm}}^{1\%} \begin{cases} 693 \text{ m}\mu & 49 \\ 617 & \text{,,} & 693 \\ 583 & \text{,,} & 404 \end{cases}$$

containing about 14% of vitamin A or c. 560 p.p.m. in the tissue.

Sample 6b. The corresponding bulk sample yielded 1.25% of oil with c. 5% of vitamin A, or 625 p.p.m. in the tissue.

Sample 6c. The mesentery from fish B yielded 2.2% of oil:

$$E_{1\text{ cm}}^{1\%} \begin{cases} 693 \text{ m}\mu & \text{Feeble} \\ 617 & \text{,,} & 169 \\ 583 & \text{,,} & 90 \\ 328 & \text{,,} & 53 \end{cases}$$

containing 3.25% of vitamin, or 734 p.p.m. in the tissue.

The data are collected for reference in Tables I and II, the most probable values being given.

Table I. *Data for fish A*

Coat	Pyloric caeca and duodenum			Ileum		
	Total wt. of tissue g.	Fat %	Vitamin A p.p.m.	Total wt. of tissue g.	Fat %	Vitamin A p.p.m.
Mucosal epithelium*	920	2.2	2,680	—	—	—
<i>Tunica propria</i>	235	11.0	33,000	38	11.0	35,750
<i>Stratum compactum</i>	164	3.1	9,000	61	1.8	3,240
<i>Stratum granulosum</i>						
Muscle	105	1.1	220	32	1.0	70
<i>Serosa</i>	124	1.2	480	27	1.55	310
Mesentery*	419	1.25	625	—	—	—

\* Material from the combined pyloric caeca, duodenum and ileum.

Table II. *Data for fish B*

Coat	Combined pyloric caeca, duodenum and ileum			
	Total wt. of tissue g.	Fat %	Vitamin A p.p.m.	Total vitamin A g.
Mucosal epithelium	468	7.5	2,590	1.21
<i>Tunica propria</i>	232	10.3	18,540	4.30
<i>Stratum compactum</i>	101	4.8	13,440	1.36
<i>Stratum granulosum</i>				
Muscle	92	1.2	187	0.017
<i>Serosa</i>	67	1.6	339	0.022
Mesentery	197	2.2	734	0.144

It is important to know how much of the vitamin A located in the intestines exists as free alcohol and how much as ester. The only satisfactory approach to



this question is to determine the elimination curve of vitamin A in the process of molecular distillation. For this experiment to be worth while, a relatively large quantity of oil must be accumulated from fresh tissues in which autolysis has not occurred to any great extent. Our first attempts to inhibit autolysis (by storage and transport in tins surrounded by ice and salt) have not been successful and at this stage we shall confine ourselves to reporting the free fatty acid contents of various preparations:

Extract from mucosal epithelium	...	...	...	...	21.0% F.F.A.
„ <i>tunica propria</i>	...	...	...	...	7.5% „
„ <i>tunica propria</i> (ileum)	...	...	...	...	5.3% „
„ <i>stratum compactum</i> and <i>stratum granulosum</i>	...	...	...	...	13.6% „

It is desirable to obtain such data on preparations from absolutely fresh tissue in order to distinguish between post-mortem hydrolytic products and such free acid as may exist in the naturally functioning tissue.

#### DISCUSSION

The outstanding experimental finding is that the vitamin A occurring in the intestines of the halibut is sharply localized in certain coats. In fish A the *tunica propria* is far richer than the other mucosal coats, whilst the latter in turn are much richer in vitamin than the muscle and *serosa*. In fish B the separation is not quite so sharp, but here also the *tunica propria* is the tissue having the highest concentration of vitamin A.

In an earlier paper [Edisbury *et al.* 1938] the view was advanced that vitamin A might assist in absorption processes (especially of fat), in view of its localization in the absorptive portions of the intestine and its relative absence from the rest of the alimentary tract. The bearing of the present results on this hypothesis may now be considered.

The parallelism between the presence of vitamin and of fat in the various tissues, noted for the alimentary tract as a whole, can be seen to hold equally well in the various intestinal coats. Even when account is taken of the fact that the "fat" percentages recorded include vitamin A esters it is true that the glycerides and the vitamin A esters exhibit parallel distributions.

Greene [1913], again working with the king salmon, has shown that the *tunica propria* and the *stratum compactum* are of peculiar significance in the process of fat absorption in fish. The intestine of a fish is less complex than that of a mammal and fat absorption appears to proceed firstly by diffusion from the lumen into the mucosal epithelium. Possibly bile acids assist in making fatty acids water-soluble, as is the current conception in the case of mammals [Verzár & McDougall, 1936; Frazer, 1938], but absorption by villi and the lymph system does not seem to occur.

In the epithelium the fat appears as droplets which stain as neutral fat. The next stage in absorption is the temporary storage of this fat in the spaces of the *tunica propria*, which acts as a kind of temporary reservoir. The *stratum compactum* forms a definite and continuous boundary to this reservoir. "It is a continuous membrane with no discernible openings other than at the points where blood vessels enter. Any fat passing through the *stratum compactum* would have to pass through in solution or else be carried within in the lumen of the blood vessel. In either case no definite fat globules as such get by this membrane from the *tunica propria*."

Thus, the fat globules in the *tunica propria* must be dispersed and rendered diffusible through the walls of the blood vessels or else through the *stratum*

*compactum* itself. It is noteworthy that fat leaves the *tunica propria* relatively slowly. It is unlikely that bile acids are present in the *tunica propria* and that fatty acids are rendered water-soluble again for diffusion. It is, however, conceivable that the large quantities of vitamin A present in these tissues may make possible the diffusion of fatty acid either through the blood vessel wall or through the *stratum compactum*.

There is evidence that vitamin A may form a complex with protein, as do certain carotenoids (e.g. astacene). Such a complex exists in visual purple and probably also in liver [Edisbury *et al.* 1938]. Such complexes may well form an integral part of the cell structure of these mucosal tissues and the vitamin A, by repeated esterification and hydrolysis, could thus assist in the transport of fatty acid.

With the evidence at present available it would be unwise to speculate further but should later evidence support this hypothesis there will still be many points to be cleared up. It will be necessary to know whether the fat leaves the *tunica propria* by the blood vessels or directly through the *stratum compactum*. The presence of large quantities of vitamin in the latter coat itself (as well as of appreciable, if much smaller amounts in the muscle, *serosa* and mesentery) suggests that at least a part of the fat may diffuse directly through the *stratum compactum*. It would also be of interest to know whether fat is transported to the liver as vitamin esters or as reformed glycerides. In those fish which do not have large quantities of vitamin A in the intestines, some other substance (e.g. cholesterol or phosphatide) may fulfil a similar function. The hypothesis we have tentatively advanced accounts for the presence of very large amounts of vitamin A in the alimentary tract of certain fishes and also suggests one way in which fat may be transported through the intestinal wall.

#### SUMMARY

The large vitamin A deposits in the intestines of many species of fish are shown by two methods, applied to halibut, to be sharply localized in the *mucosa* and in particular in the *tunica propria*.

The distribution of fat in the intestine runs parallel with that of vitamin A.

Absorption of fat by fish differs from that obtaining in mammals, proceeding directly through the mucosal epithelium and not by a lymphatic system. The existence of a dense, continuous layer of connective tissue (*stratum compactum*) forms a barrier to fat transport as droplets, which accumulate in the adjacent *tunica propria*.

The hypothesis is advanced that vitamin A esters, in combination with protein, may assist in the dispersion of these droplets and in the removal of fat from the *tunica propria*.

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## XLII. HALIBUT INTESTINAL OIL

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HALIBUT intestines have been found to autolyse very rapidly. The fat present in any but the freshest material may therefore have undergone considerable hydrolysis. Oil from stale tissue contains more free acid than that from comparatively fresh material, but so far neutral oils have not been obtained. Hitherto, it has not been possible to carry out extraction sooner than 2 or 3 days after the death of the fish. Preservation in ice and salt certainly slowed down autolysis, but special experiments will be necessary before the free fatty acid content of the functioning intestine can be determined.

A beginning has been made towards ascertaining, (a) the proportions of free and combined vitamin A, (b) the nature of the fatty acids present in the vitamin esters, (c) the relative speeds of enzymic hydrolysis of vitamin esters and glycerides and, (d) the extent to which autolysis results in contamination of the "fatty" extract.

The method of molecular distillation applied to oils [Hickman, 1937, 1] permits the approximate determination of free and combined vitamin A provided the oil is not excessively acid and some 100 ml. are available. The use of constant yield oil as solvent [Hickman, 1937, 2] extends the method to small quantities of solute.

In 1937 a sample of oil extracted from the ileum of a relatively fresh halibut was subjected to analytical distillation using Hickman's constant yield oil. 0.5 g. was diluted with 75 ml. constant yield oil and 75 ml. of residue oil from a previous molecular distillation. Fractions were collected (10° temperature and 18 min. time intervals) and vitamin yields were determined by assaying the whole series. About 4% of the total vitamin A occurred in the fractions collected over the range 90–140° and 96% was volatile at higher temperatures. The elimination curve over the range 150–240° was tall and narrow with a maximum near 210°. The results indicate that approximately 95% of the vitamin occurred as esters and that the acids which were combined in the greater part of these did not differ much in molecular weight.

An attempt was made in the autumn of 1937 to collect sufficient halibut intestines at Aberdeen for a distillation of the undiluted intestinal oil. Unfortunately, the material as received from the fishing vessels had already undergone considerable autolysis, so that the oil obtained (150 g.) had a high acid value (92.4).

A trial distillation gave the results shown in Table I.

About 20–30% of the vitamin existed in the free state and the free acids accompanied the vitamin in the distillates. The high acid content facilitated cyclization and made it difficult to collect the distillates without overheating the condenser.

Accordingly, the remainder of the oil was washed with aqueous  $K_2CO_3$ , the oil being first dissolved in ether and the amount of  $K_2CO_3$  calculated from the

Table I. *Molecular distillation of 53 g. halibut intestinal oil. B.V. 8300. Acid value 92.4*

Fraction	Temp. ° C.	Wt. (g.)	B.V.	Acid value	Colour test		u.v. absorption
					$E_{1\text{ cm}}^{1\%}$ 620 m $\mu$	$E_{1\text{ cm}}^{1\%}$ 580 m $\mu$	$E_{1\text{ cm}}^{1\%}$ 325 m $\mu$
1	100-120	8.2	6400	156	454	230	113*
2	130-140	12.6	6200	129	400	210	98†
Residue	—	32.2	9000	47	715	365	204‡

\* Fine structure due to cyclization very marked.

† Fine structure less marked.

‡ No fine structure.

acid value. The acid value was reduced from 92.4 to 37 and although a lower acidity would have been desirable the oil was distilled at this stage lest further washing with alkali should change the proportion of free to esterified vitamin.

85 g. of washed oil mixed with 15 g. of residual oil were distilled and 15 fractions were collected.

 Table II. *Molecular distillation of washed halibut intestinal oil. B.V. 17,000. Acid value 37*

Frac- tion	Temp. C.	Wt g.	Carry- Price B.V.	u.v. absorp- tion $E_{1\text{ cm}}^{1\%}$ 325 m $\mu$	Colour test, $E_{1\text{ cm}}^{1\%}$				Non- sap. %	Recovered acids	
				620 m $\mu$	580 m $\mu$	693 m $\mu$	%	%		Mol. wt.	
1	90	2.8	17,300	200*	875	480	< 50	—	—	—	
2	100	4.4	17,300	280*	1185	616	< 47	—	—	—	
3	110	4.2	17,700	188†	880	490	< 42	—	—	—	
4	120	5.0	14,250	255†	1100	577	< 58	—	—	—	
5	130	4.7	10,500	180†	725	400	< 43	—	—	—	
6	140	5.2	6,380	112.5	337	172	—	53.5	46.9	316	
7	150	3.6	6,120	116.6	306	160	—	65.6	37.45	315	
8	160-70	7.6	15,676	300	1140	627	—	—	—	—	
9	180	3.1	29,000	500	1900	986	93	—	—	—	
10	190	2.4	33,300	530	1880	1000	97	55.5	32.8	276	
11	200	4.1	29,000	590	2080	1075	113	51.1	45.6	280	
12	210	4.7	33,300	550	2006	1060	125	50.7	45.9	278	
13	220	1.3	31,500	482	1875	990	93	48.3	51.25	284	
14	230-40	9.4	17,300	440	1200	676	58	48.0	52.3	291	
Residue	—	34.5	2,900	90†	211	156	13.4	28.7	68.05	301	

\* Fine structure marked.

† Feeble fine structure.

‡ Anomalous absorption.

From Table II it would seem that about 26 % of the total vitamin A in this sample of oil occurred as free alcohol. The ratio  $E_{1\text{ cm}}^{1\%}$  325 m $\mu$ /620 m $\mu$  (ultra-violet absorption to absorption for the main band in the colour test) is curiously variable. For the richest preparations of vitamin A prepared by saponification but not subjected to molecular distillation the ratio is about 0.32 and for the purest distillates of the free vitamin perhaps 0.30. In the richest undistilled esters which we have prepared from intestinal oils (60-70 % vitamin esters) this ratio varies from 0.29 to 0.32.

Now the cyclization product of vitamin A is itself chromogenic towards  $\text{SbCl}_3$  and it is striking that for fractions 1-5 the ratio  $E_{1\text{ cm}}^{1\%}$  325 m $\mu$ /620 m $\mu$  varies from 0.21 to 0.25. The cyclized product may well account for the main discrepancies. Fractions 6 and 7 are the first to show no spectroscopic evidence of cyclized vitamin A, and the ratio jumps to 0.33 and 0.38. Fractions 8 and 9 show an unusually low ratio for vitamin esters, whilst Fractions 10, 11 and 12

are much more nearly normal. It is interesting that much of the non-saponifiable other than vitamin A distils mainly between 140° and 180°. The discrepancies may indicate variable proportions of stereoisomerides of vitamin A in the different fractions, but other explanations are equally plausible.

A similar distillation carried out on a fish liver oil affords a useful comparison :

Table III. *Molecular distillation of a sturgeon liver oil\**

Frac- tion	Temp. ° C.	Vitamin A %	Ratio $E_{1\text{ cm}}^{1\%}$ 325 m $\mu$ 620 m $\mu$	Vitamin esters %	Non- sap. %	Recovered acids		Cyclized vitamin A %	Non-sap. other than vitamin A and spurious A %
						%	Mol. wt.		
Original oil		7.9	0.305	—	24.7	69.5	278.5	None	17
1 (not retained)									
2	100	11.2	0.26	—	27.4	65.0	266	13-14	c. 16
3	110	15.1	0.26	—	34.7	57.7	272	< 14	c. 19.6
4	120	15.8	0.26	—	38.5	56.0	273	< 10	c. 23
5	130	16.4	0.30	—	43.5	52.0	277	Very little	27
6	140	14.3	0.29	—	49.8	44.8	278	None	35.4
7	150	11.5	0.31	21	55.0	38.7	287	..	43.5
8	160	9.7	0.30	19	67.7	33.1	293	..	58
9	170	11.8	0.30	23	64.1	33.8	297	..	52.2
10	180	21.4	0.30	40	64.5	37.2	280	..	43.1
11	190	29.5	0.28	57	56.1	44.7	276	..	27
12	200	31.6	0.29	60	51.4	50.8	273	..	20
13	210	29.0	0.28	55	47.8	51.3	270	..	18.8
14	220	29.4	0.29	42	44.2	57.2	279	..	20.9
15	230	15.9	0.31	30	39.5	61.2	278	..	23.6
16	240	9.1	0.30	17.5	32.9	66.4	277	..	23.8
17	250	4.7	0.31	c. 9	25.5	72.3	272	..	20.8
Residue		Very low							

\* Part of the vitamin A esters had probably been hydrolysed in the process of extraction.

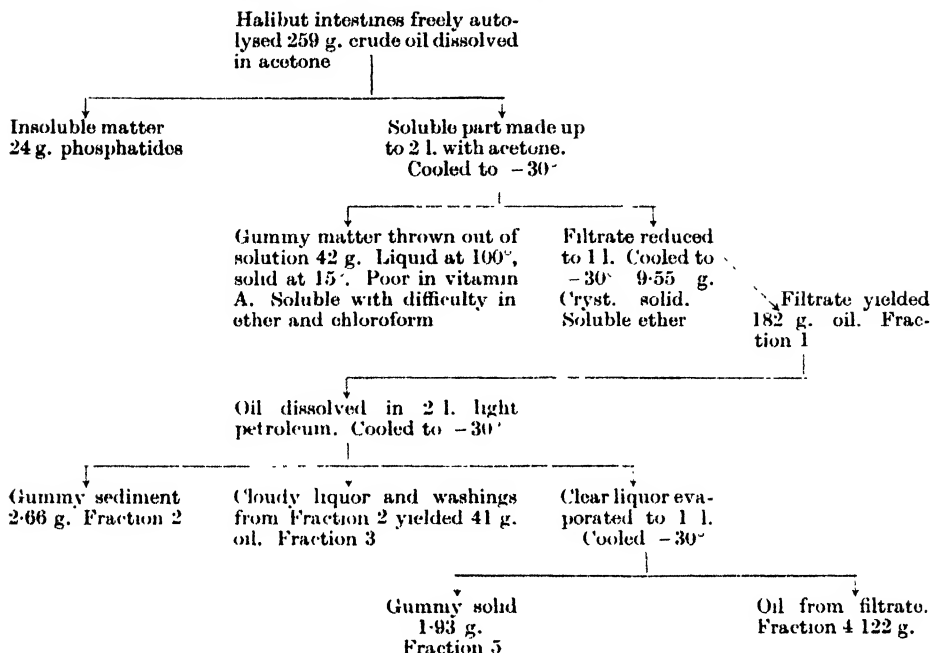
These results indicate a very constant ratio between ultraviolet absorption and colour test intensities once the free acids and cyclized vitamin A have been eliminated. They also illustrate very clearly the limitations imposed by the potency of the original oil and its high content of sterol and sterol esters. Fractions containing 60% of vitamin esters are obtained but the overlap which exists between the elimination curves of vitamin esters and sterol esters sets a limit to the efficacy of the process. The chances of preparing richer ester fractions would be greatly enhanced if sufficient neutral intestinal oil of high potency (60-70% vitamin esters as already described) could be obtained. Unfortunately, the bulk of the halibut recently accessible to us has consisted mainly of smaller fish and it has been impossible to obtain fresh intestines. The next step will be to arrange for better preservation on the fishing vessels.

In the meantime experiments have been carried out on the material available.

Table IV. *Fractionation of oil from autolysed intestines*

Fraction	Non-sap. %	Recovered acids		Colour test, $E_{1\text{ cm}}^{1\%}$			Ultraviolet, $E_{1\text{ cm}}^{1\%}$	
		%	Mol. wt.	693 m $\mu$	620 m $\mu$	580 m $\mu$	325 m $\mu$	261 m $\mu$
1	21.7	61.7	292	31.5	525	283	158.3	—
2	—	—	—	6.93	110	67.5	38.2	52
3	22.3	65.1	297	29.6	490	286	158	—
4	22.1	70.2	297	32.9	537	302	158.3	—
5	—	—	—	6	27.5	19	11.7	26.6

The following diagram illustrates the scope of the work:



Attention may be called to the following points:

- (1) The original extract contained about 25% of material which was not glyceride, nor vitamin ester, nor even mainly sterol ester.
- (2) Even after the acetone treatment further small quantities of material containing relatively little vitamin can be eliminated.
- (3) The process, however, is still far from complete. Thus, the "non-sap," and the acids recovered show a large deficit from 100% in Fraction 1. This is diminished in Fractions 3 and 4 (Table IV).
- (4) The gummy residues exhibit a new and well defined absorption band at  $261\text{ m}\mu$  (Fractions 2 and 5) which is not shown and may be masked in the other fractions. The new absorbing material remains in the aqueous liquor after acidifying the soaps with  $\text{H}_2\text{SO}_4$  and extracting with ether. It can be extracted from the evaporated residue by means of alcohol but is accompanied by other products.

The whole problem of the occurrence of substances other than glycerides, vitamin esters and sterol esters in "oils" is under investigation and it seems wise to defer the more detailed discussion of the problems raised above. The molecular distillations provide data for constructing elimination curves for vitamin A and vitamin  $\text{A}_2$  [cf. Hickman, 1937, 1, 2].

Fig. 1 shows  $E_{1\text{ cm}}^{1\%}$  plotted against temperature for the ultraviolet absorption. The elimination curve shows two maxima, one corresponding with the free vitamin and the other with the esterified vitamin. Fig. 2 shows similar curves for the 693, 620 and  $583\text{ m}\mu$  bands in the colour test. It is true that the oil contains much more vitamin A than vitamin  $\text{A}_2$  and that the proportions are not altogether favourable for a separation. Nevertheless, the similarity between the elimination curves for the two vitamins is very striking and is a sign that the

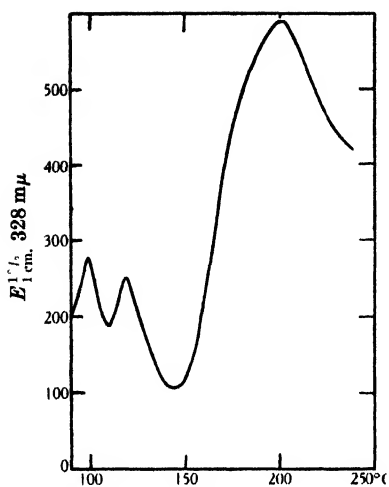


Fig. 1.

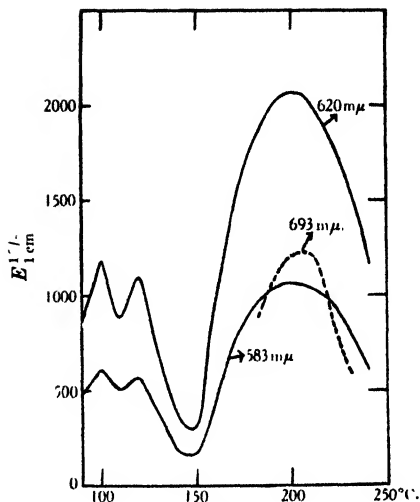


Fig. 2.

Fig. 1. Halibut intestinal oil. The potency of each molecular distillate as measured by r.v. absorption has been plotted against the temperature of distillation. The smaller maxima are due to free vitamin A and the cyclization product, the large maximum to esterified vitamin A.

Fig. 2.  $\text{SbCl}_5$  colour test data for halibut intestinal oil distillates are plotted against temperature. The 693  $\text{m}\mu$  maximum concerns vitamin  $\text{A}_2$ , the 620  $\text{m}\mu$  and 583  $\text{m}\mu$  maxima vitamin A.

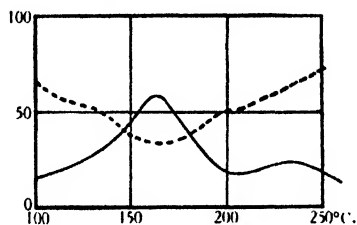


Fig. 3. The non-saponifiable fractions and the acids recovered from the soaps of halibut intestinal oil distillates were obtained and vitamin A estimated spectrographically. — % non-sap. --- % recovered acids.

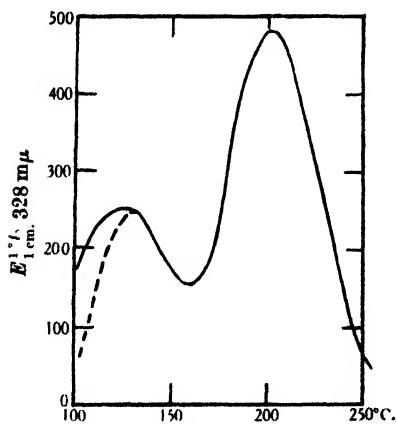


Fig. 4.

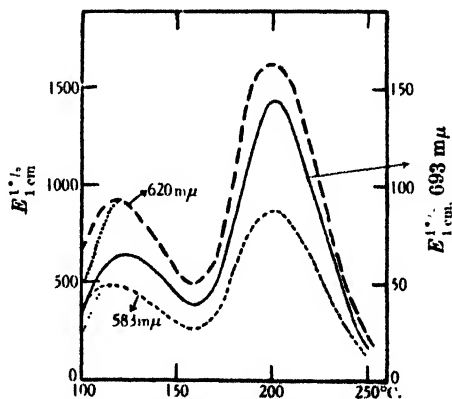


Fig. 5.

Figs. 4 and 5 are comparable with Figs. 1 and 2 and apply to sturgeon liver oil distillates. The dotted portions indicate the course of the curve if no cyclization had occurred.

structure of vitamin A<sub>2</sub> cannot be very different from that of vitamin A. The similarity between the 620 m $\mu$  and 583 m $\mu$  bands, which both appear to be due to vitamin A, persists after molecular distillation. The elimination curves indicate that both bands are mainly if not entirely due to vitamin A. The halibut intestinal oil which was distilled contained more of the cyclized vitamin than the liver oil used. Cyclization, which is presumably caused by the heating process, is greatly accelerated in the presence of free fatty acid. The elimination curves show quite clearly the extent to which free vitamin A and its cyclization product can be separated. Most of these findings confirm the work of Hickman and his associates. The failure to separate vitamin A from vitamin A<sub>2</sub> is surprising. The work should be repeated on the liver oil from freshwater fish in which vitamin A<sub>2</sub> predominates over vitamin A, and the conditions may prove favourable to the detection of the small differences in the elimination curves if the two vitamins are homologous.

#### SUMMARY

1. Vitamin A in halibut intestinal oil exists almost entirely (at least 95%) as esters.
2. Halibut intestines autolyse freely so that the extractable "oil" is a mixture of free fatty acids, free cholesterol and free vitamin A with their esters. The oil is also contaminated with phosphatides and other products. Among these is a water-soluble material showing selective absorption  $\lambda_{\max}$  261 m $\mu$ . The study of this substance is being pursued.
3. It is difficult to remove free acids from the autolysed material and cyclization occurs to an appreciable extent on distillation.
4. The role of impurities in affecting the physical assay of vitamin A in rich materials is illustrated and, to some extent, accounted for.
5. The elimination curve of vitamin A<sub>2</sub> appears to resemble that of vitamin A very closely.

Our thanks are due to the Medical Research Council for a grant in aid of the expenses of this work (R. A. M.), and to Dr F. H. Carr of British Drug Houses for granting facilities for the molecular distillations.

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# XLIII. THE PHYSIOLOGICAL PROPERTIES OF ASCORBIC ACID

## II. THE INCREASE IN METABOLISM OF GUINEA-PIGS ON A DIET DEFICIENT IN ASCORBIC ACID

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It was reported [McHenry *et al.* 1938] that, when two similar groups of guinea-pigs were pair-fed, one group receiving ascorbic acid while the other did not, there was a significant loss in weight in the deficient group at the end of three weeks. In seeking an explanation for this loss in weight, the metabolic rates of the two groups were investigated.

Although the basal metabolism of guinea-pigs suffering from scurvy has been determined by several investigators, conflicting results have been obtained. Söderström & Törnblom [1933], Soneda & Kato [1934], Törnblom [1934], Ardy & Bellini [1936], Fujino [1936] and Scoz *et al.* [1937] all reported that the O<sub>2</sub> consumption in experimental scurvy decreased until death. On the other hand, Mosonyi & Rigo [1933] and Calcinai & Galigani [1934] found a markedly increased O<sub>2</sub> consumption in scorbutic animals.

### *Methods*

In order that the animals in our experiments might be similarly fed, the technique of paired feeding was employed [McHenry *et al.* 1938]. It consists, briefly, in fixing the quantity of food of the ascorbic acid group by the amount of the basal diet consumed by the scorbutic group. In this manner an isocaloric intake was maintained in the two groups. The minimal survival time of the animals on the deficient diet was 21 days, but the metabolic rate was taken on the 18th day. The composition of the basal diet and the care of the animals were similar to those previously reported. Guinea-pigs receiving ascorbic acid were given 5 mg. per day by mouth, a dose which is adequate for growth.

For the determination of the gaseous exchange the respiration apparatus used was that of Macleod *et al.* [1929] with some recent modifications. Although originally planned for dogs it could be used for a group of small animals such as rats and guinea-pigs where the combined volume of the gaseous exchange was sufficiently great to minimize the error. There was no method for recording the activity of the small animals, but in some earlier work with rats it had been found that in a run of about 6 hr. following a 16-hr fast, an average of the two lowest hourly rates gave a figure suitable for comparative purposes even though no record of activity was obtained. It was not always convenient to have the temperatures at that of "thermal neutrality" for the small animals, but when groups were to be compared the average temperature of the cabinet was within a fraction of a degree of the same temperature.

*Experimental results*

Results were obtained in three series of experiments, the first with two groups of 6 and 8 animals each, the second with two groups of 10 animals each and the third with two groups of 10 animals each together with a normal control group of 10 receiving basal diet *ad lib.* plus ascorbic acid. The  $O_2$  consumption is given on a body-weight basis and the heat output calculated on a body-surface basis in the hope of correcting, to some extent, the errors due to weight differences. The formula used for calculating body surface was  $S = 8.9 \times W^{\frac{2}{3}}$  where  $W$  = weight after the metabolism was measured. For the determination of heat production the caloric equivalent of the  $O_2$  consumption was derived from the R.Q. by the usual table. The highest R.Q. was 0.81, the lowest 0.74, but in the majority of the hourly periods the range was between 0.75 and 0.78. There were no significant differences between groups.

Metabolic rates obtained on the three series are given in Table I. Dates of the experiments are given because of the fact that there is possibly an alteration in guinea-pigs from season to season; at least this applies to resistance to infection or to bacterial toxins.

Table I

Date	Group	No. of animals	Sex	Diet	$O_2$ /kg./hr. ml.	Diff. %	Cal./m <sup>2</sup> / 24 hr.	Diff. %
26-27 Apr.	Paired	6	2 M., 4 F.	Basal + ascorbic acid	925	—	755	—
	Scorbutic	8	4 M., 4 F.	Basal <i>ad lib.</i>	1206	+23	941	+19
31 May- 1 June	Paired	10	—	Basal + ascorbic acid	979	—	793	—
	Scorbutic	10	—	Basal <i>ad lib.</i>	1282	+30	988	+19
	After 40 mg. of ascorbic acid				1192	—	916	—
18-20 Oct.	Normal control	10	7 M., 3 F.	Basal <i>ad lib.</i> + ascorbic acid	1093	—	925	—
	Paired	10	7 M., 3 F.	Basal + ascorbic acid	1036	—	872	—
	Scorbutic	10	7 M., 3 F.	Basal <i>ad lib.</i>	1366	+24	1096	+20

There is no record of the sex of animals in the second series but, as in the other two, they were mixed, the two groups being identical in this respect. In the second series an effort was made to demonstrate that the rise in metabolic rate was due specifically to a deficiency of ascorbic acid by giving the deficient animals a total of 40 mg. ascorbic acid and repeating the determination. The metabolism was lowered somewhat and, in all probability, had the treatment been continued, would have returned to normal.

In the third series the normal control group shows that the pair-fed animals receiving ascorbic acid have a metabolism slightly below normal but close enough to be taken as normal in comparison with scorbutic ones.

## DISCUSSION

These three sets of experiments carried out in two seasons of the year have shown that guinea-pigs on a diet which though deficient in ascorbic acid is otherwise adequate, exhibit a definite increase in metabolism. Since paired feeding was employed, the results are due to a single variable, namely, the absence of ascorbic acid.

The fact must not be ignored, however, that the calculation of a metabolic rate after a loss in weight can, under certain conditions, be quite misleading.

Should the loss in weight be due to decrease of inert substances such as water and fat while active tissue remains the same in all respects, then the metabolic rate, if based on unit of weight, will appear to rise as the weight falls, although the animal is consuming the same amount of  $O_2$  throughout. A rate in terms of surface area tends to reduce this error, and although some workers maintain that there is no causal relationship between surface area and heat production, this method of expression continues to be employed. Nevertheless, since the formulae ordinarily used for obtaining the surface area of laboratory animals are based entirely on weight, the objection remains that there is no distinction between inert material and active tissue, and therefore increases in metabolic rate associated with weight-loss or decrease in rate accompanying a weight-gain should be carefully considered.

If it be assumed, though quite unjustifiably, that the changes in weight are due solely to fluctuations of inert material, then the weights at the beginning of the experiment might be used as the basis of calculation. Even on this assumption the scorbutic groups show increases in metabolic rate of 13, 13 and 12 % respectively in the three series of experiments. Also, the ratios of  $O_2$  consumed to the amounts of dry material found in the bodies of the animals at the end of the experiment confirmed the conclusion that the rise in metabolic rate was not due to loss of water. Under these circumstances there is no need to depart from the conventional form of expression of the metabolic rate as calories per square metre of body surface. Comparisons can then be made with other work.

A number of investigators have suggested that there is a relation between the intake of ascorbic acid and the activity of the thyroid gland. For example, Mosonyi [1936] states that the increase in metabolism observed by him is due to hyperthyroidism, but as yet we have no experimental evidence on which to base such a conjecture.

#### SUMMARY

Guinea-pigs on a basal scorbutic diet show, on the 18th day of this regimen, a greater metabolic rate than do animals with the same food intake but receiving ascorbic acid.

Grateful thanks are expressed to the Banting Research Foundation for a grant to one of the authors (M. S.).

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# XLIV. STUDIES ON DIFFUSING FACTORS

## II. COMPARISON OF DIFFUSING FACTORS FROM DIFFERENT SOURCES AND PREPARATION OF CONCENTRATES FROM BULL TESTICLE

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It has been recently suggested [Christensen, 1938] that kallikrein, the non-dialysable vasodilator principle from pancreas and urine, is a diffusing factor similar to that in mammalian testicle. This suggestion was based on the fact that intracutaneous injections of kallikrein, mixed with a suitable indicator, result in the spread of the latter over a much larger area than in the case of control injections of indicator alone. The areas were compared 24 hr. after injection.

In endeavouring to compare the relative spreading activities of kallikrein preparations and diffusing factor concentrates, some differences between the modes of action of the two substances have been observed. The technique used for the comparison was that recently described [Madinaveitia, 1938] in which haemoglobin is used as an indicator of the area of skin penetrated by the diffusing factor. The characteristic effect of the testicular diffusing factor is the immediate flattening of the blebs formed by intracutaneous injection of the solution. When haemoglobin is present in the injected fluid the visible effect is a rapid increase of the area of skin coloured by haemoglobin (Figs. 1, 2, 3, curve *DF*).

The effect of intracutaneous injections of kallikrein is different. The skin first becomes oedematous at the site of injection and the oedema slowly spreads. The weal takes a long time to disappear. When haemoglobin is present in the injected fluid it slowly diffuses through this oedematous area (Figs. 1, 2, 3, curve *K*). The area of skin over which the haemoglobin spreads eventually becomes equal to, and even larger than, that following injection of testicular diffusing factor. After some hours the oedema disappears leaving a patch of skin coloured by haemoglobin, identical in appearance with that obtained by injection of testicular diffusing factor. Although the final result as regards the spread of haemoglobin is identical in both cases, the ways in which it is obtained are different. It seems therefore that the testicular diffusing factor and kallikrein are not identical in their biological actions. The available data on diffusing factors, other than the testicular one, are mainly based on measurements taken at least 24 hr. after injection. It has been found desirable to establish whether their actions are like that of the testicular diffusing factor or like that of kallikrein. Leech extracts, rattlesnake venom, solutions of diazobenzenesulphonic acid-serum globulin, *Clostridium Welchii* filtrates, *Vibrio septique* filtrates and staphylococcal toxoid behave, in different degrees of intensity, in a manner similar to the testicular diffusing factor (Figs. 2 and 3).

The mechanism by which the diffusing factors induce water to spread so rapidly through the skin tissues is not yet known. This spread is observed when injections are made in the isolated skin of a rabbit (Fig. 4; see also McClean [1931]). This would indicate that the mechanism by which the diffusion of water takes place through the skin tissues is independent of the circulation of

blood through the skin capillaries. A further support for this view might perhaps be found in the fact that the presence of adrenaline or of histamine in the injected fluid does not interfere with the action of testicular diffusing factor. Neither of these substances has any spreading activity.

Purified preparations of diffusing factor are devoid of haemolytic properties [Favilli & McClean, 1934]. This, together with the fact that substances like saponin, bile salts [McClean, 1930], triethanolamine, soap, allylamine and octyl alcohol do not show the characteristic spreading properties of the testicular diffusing factor, indicates that the spreading is not due to a surface tension effect.

It seems that the diffusing factor is able to increase the porosity of the skin tissues. The increase in the diameter of the pores appears to be substantial, since through them can pass particles as large as those of Indian ink.

Most of the available evidence on the chemical constitution of the testicular diffusing factor suggests that it is a protein, or an active constituent intimately associated with a protein carrier. Nevertheless, some earlier observations did not agree with this point of view. In the preparation of concentrates by fractional lead acetate precipitation [Morgan & McClean, 1932; Madinaveitia, 1938] most of the proteins are removed by precipitation with neutral lead acetate, the active material remaining in the filtrate. Since most proteins precipitate under these conditions it seemed doubtful whether the diffusing factor was a protein or not. Another point which did not seem to be in agreement with the diffusing factor being a protein is the fact that some of the preparations made by Aylward [1937] did not coagulate on heating.

The fractionation of aqueous testicular extracts with  $(\text{NH}_4)_2\text{SO}_4$  has shown that these two facts do not exclude the possibility of the diffusing factor being a protein or being intimately associated with one. Only a small amount of active material precipitates at half saturation with  $(\text{NH}_4)_2\text{SO}_4$ , and under the conditions used precipitation is not complete at full saturation (Figs. 5 and 6). From a half-saturated solution the  $(\text{NH}_4)_2\text{SO}_4$  is easily removed by dialysis. The resulting solution retains the full activity of the original extract and contains only 1.6 % of the N present in the desiccated bull testicle powder. Half saturation with  $(\text{NH}_4)_2\text{SO}_4$ , removal of the precipitate and dialysis of the filtrate give a purification of the same order as that obtained by the fractional lead precipitation method.

McClean [1936], working with diffusing factor from bacterial filtrates, found that it was completely precipitated at full saturation with  $(\text{NH}_4)_2\text{SO}_4$ ; Claude & Duran-Reynals [1937] came to the same conclusion. These authors, before treatment with  $(\text{NH}_4)_2\text{SO}_4$ , precipitated the active material with acetone and extracted this precipitate with water. This procedure denatures the proteins; only 1-10 % of the original activity is recovered from the acetone precipitate.

Preparations obtained by  $(\text{NH}_4)_2\text{SO}_4$  fractionation do not give a precipitate with lead acetate, basic or neutral. They readily precipitate with other protein precipitants. The peculiar failure of these proteins to precipitate with lead acetate explains the mechanism of the purification of diffusing factor by means of the fractional lead precipitation. Half saturation with  $(\text{NH}_4)_2\text{SO}_4$  and subsequent dialysis of the filtrate yields a solution of proteins which are not precipitated by lead acetate.

The heat-coagulation of purified  $(\text{NH}_4)_2\text{SO}_4$  diffusing factor is greatly dependent on the pH at which the solution is heated. Between pH 5 and 6 coagulation was observed, but at other pH the solution remained clear after heating. This would indicate that the isoelectric point of this preparation is somewhere between pH 5 and 6. No indication is given by previous workers

about the pH at which they heated their solutions and it might very well be that some of them were not in the optimum range.

The active constituents of crude testicular extracts cannot be separated from proteins by chromatographic analysis. Some of the proteins present in the extracts submitted to adsorption were less readily adsorbed than the active constituents, and others much more. The separation thus achieved was not so complete as that attained by  $(\text{NH}_4)_2\text{SO}_4$  precipitation.

#### EXPERIMENTAL

##### *Comparison of testicular diffusing factor with kallikrein*

As a source of kallikrein the commercial preparation "Padutin" (Bayer) was used. One unit is stated to correspond to 0.003 mg. of a standard preparation. 9 ml. of "Padutin for oral administration" (1 ml. corresponds to 7 units) were concentrated *in vacuo* over  $\text{H}_2\text{SO}_4$  to 5.5 ml. A thick syrup resulted. In the shaved back of each of a group of three rabbits two injections of 0.3 ml. of each

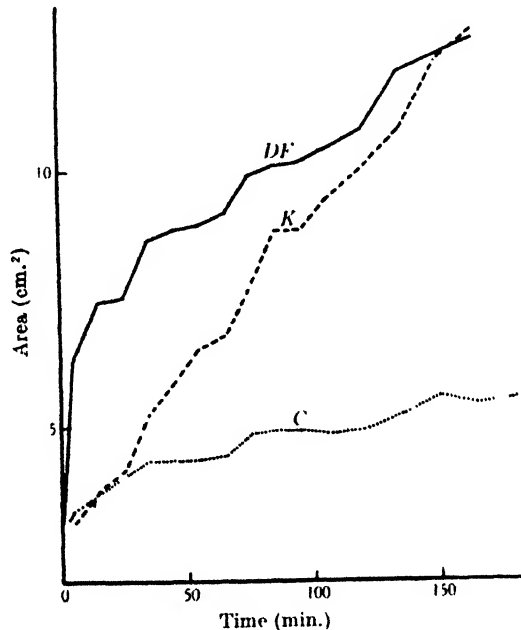


Fig. 1. Comparison of diffusing factors from different sources. C, control; W, *Clostridium Welchii*; DF, testicular diffusing factor; S, *Vibrio septique*; K, kallikrein; R, rattlesnake venom; T, *Staphylococcus*; L, leech extract; G, diazobenzenesulphonic acid-serum globulin.

of the following solutions were made: the concentrated kallikrein solution, a solution of a standard preparation of testicular diffusing factor [Madinaveitia, 1938] having 300 units/ml. and isotonic saline. All three solutions were diluted with 2 vol. of isotonic haemoglobin solution.

Measurements of the size of the coloured areas of skin were made every 10 min. during the first 2 hr. after injection and every 15 min. during the 3rd hr. The size recorded in every instance represents the average of three consecutive readings, the taking of which occupied not more than 5 min. The average of the results obtained in the three rabbits is shown graphically in Fig. 1.

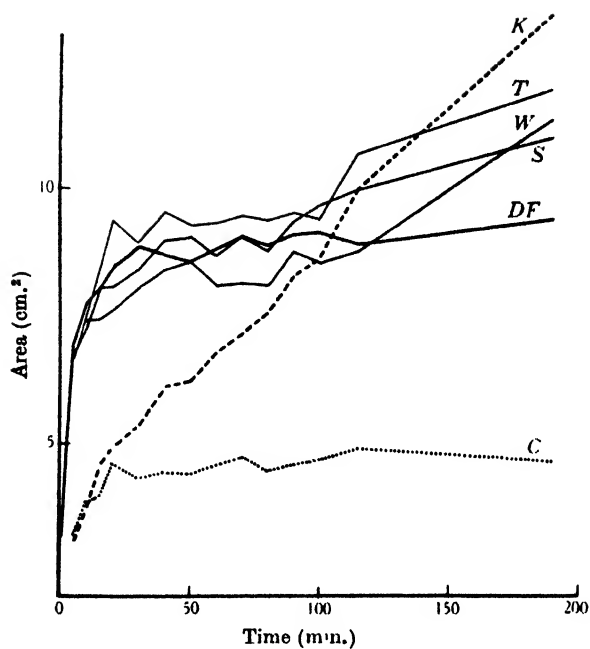


Fig. 2. For description see Fig. 1.

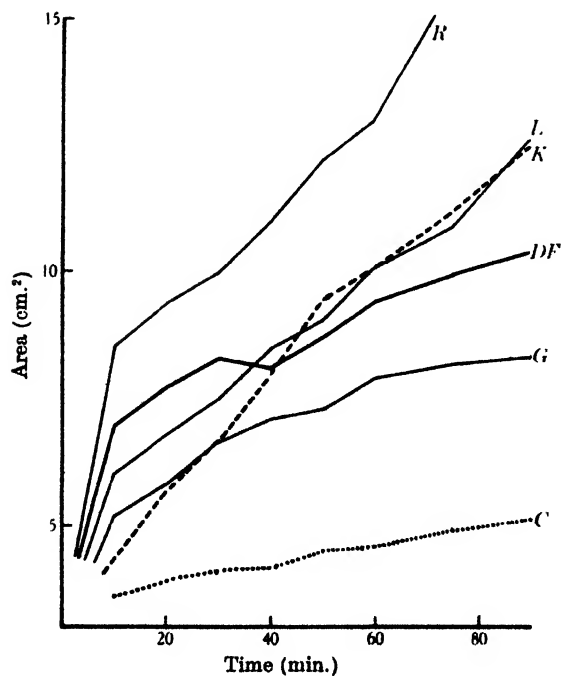


Fig. 3. For description see Fig. 1.

*Comparison of diffusing factors from different sources*

The bacterial diffusing preparations compared were filtrates of cultures of *Clostridium Welchii*, *Vibrio septique* and *Staphylococcus toxoid*, diluted with 2 vol. of an isotonic solution of haemoglobin. 0.3 ml. of each solution was injected into the shaved back of two rabbits. Injections were also made of 1:3 dilutions in isotonic haemoglobin solution of concentrated kallikrein solution (see above), a solution of testicular diffusing factor (100 units/ml.) and isotonic saline. Fig. 2 shows the rate of spread of haemoglobin in each case. The measurements were made as described in the previous experiment.

In another group of three rabbits the following solutions were compared in a similar way: diazobenzenesulphonic acid-serum globulin (kindly supplied by Prof. C. R. Harington) containing about 0.8% protein, a filtered leech extract prepared by grinding up four leeches in a mortar with 25 ml. of water, and a solution of rattlesnake venom containing 30 mg. dry venom per ml. Injections of solutions of diffusing factor (1000 units/ml.) and of a concentrated solution of kallikrein were also made, as well as a control injection of isotonic saline. The average of the results obtained in the three rabbits is represented in Fig. 3.

*Action of testicular diffusing factor on the isolated skin of a rabbit*

The back of a rabbit was shaved and the animal killed and skinned. The skin was fixed on a board over wet cotton wool. Into this prepared skin injections of 0.3 ml. of serial tenfold dilutions (in isotonic haemoglobin solution) of the

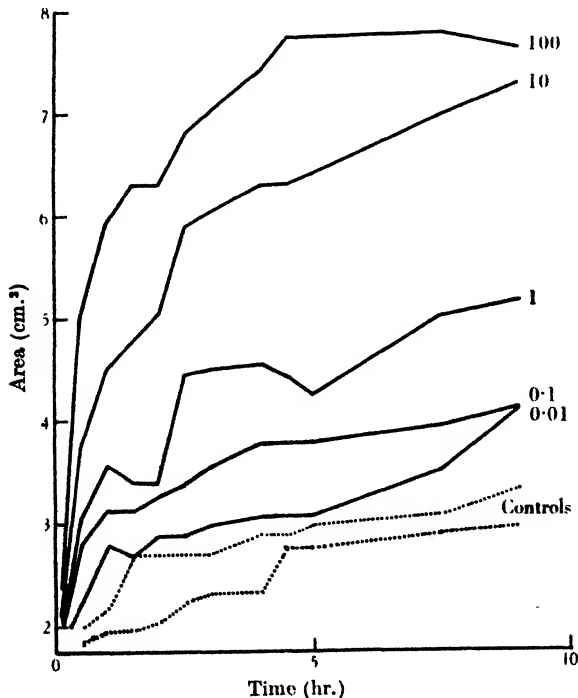


Fig. 4. Increased spread of haemoglobin in the isolated skin of a rabbit.

standard preparation were made. The most concentrated had 100 units/ml. and the most dilute 0.01 unit/ml. Two control injections of saline were also made.



The increase in the area of skin in each case stained by haemoglobin is represented in Fig. 4. Since only one animal was used in the experiment the curves are not so continuous as when the average of results in several animals is represented. The temperature of the skin during the experiment was 18–20°.

*Fractionation of aqueous extracts of bull testicle with  $(\text{NH}_4)_2\text{SO}_4$*

1 g. of dry testicle powder mixed with the same bulk of silver sand was extracted in a mortar with 20 ml. of water. After centrifuging the turbid supernatant liquor was flocculated by addition of 0.15 ml. 2*N* acetic acid. Centrifuging now yielded a clear solution with the full activity of the original material.

Increasing amounts of saturated  $(\text{NH}_4)_2\text{SO}_4$  were added to this solution as indicated below:

% of saturation	20	33	50	75	90
ml. testicular extract	4	2	2	2	1
ml. sol. sat. $(\text{NH}_4)_2\text{SO}_4$	1	1	2	2	9

The precipitate in each case was separated by centrifuging and washed with 5 ml. of  $(\text{NH}_4)_2\text{SO}_4$  of the corresponding concentration. Both the precipitates and supernatants of each precipitation were made up to ten times the original volume of testicular extract used. All solutions were diluted with 10 vol. isotonic haemoglobin solution. Their spreading activities were compared with that of serial tenfold dilutions of the testicular extract. A group of two rabbits was used for the precipitates and another group for the supernatants. The average of the results is plotted in Figs. 5 and 6. The precipitate obtained at 50% saturation with  $(\text{NH}_4)_2\text{SO}_4$  has less than 10% of the original activity. At 75 and 90% of saturation the precipitation is still incomplete, both precipitate and supernatant having more than 10% of the activity of the original solution.

For the preparation of purified concentrates of diffusing factor the following procedure has been used: 250 g. of a dry testicle powder (5% moisture, 12.5% ether-soluble materials 11% N) were extracted overnight in a ball mill with 1 l. of water. A small amount of toluene was added as an antiseptic. After dilution with 1 l. of water 10 ml. 3*N* acetic acid were added and the mixture filtered after 1 hr. The residue was extracted during 4 hr. with 1 l. of water, filtered, and the volume of the united filtrates reduced *in vacuo* at 20° to 250 ml. This concentrated solution contains 58 mg. N/ml. (53% of the N present in the dry testicle powder). To it 1 vol. of saturated  $(\text{NH}_4)_2\text{SO}_4$  was added and after 1 hr. the precipitate removed. The clear solution was then dialysed against running tap water for 1 day, concentrated *in vacuo* at 20° at about 300 ml. and again dialysed for a day. After making up the volume of the solution to 400 ml. it contained 2.2 mg. N/ml. (i.e. 1.6% of the N present in the 250 g. dry testicle powder).

The solution so obtained has the full activity of the original testicular extract. It gives all the usual precipitation reactions of proteins, except that it is not precipitated by lead acetate, basic or neutral.

The effect of heat on the coagulation of this solution is recorded in the table below, 2 ml. of solution were heated with 10 ml. of buffer in a water bath.

M/3 acetate buffer of pH	3	4	5	6		
M/15 phosphate buffer of pH	.	.	.	6	7	8
Temp. ° C.						
70	C	C	C	C	C	C
75	C	C	T	T	C	C
80	C	C	T	T	C	C
90	C	C	T	F	C	C
100	C	C	F	F	C	C

C, clear; T, turbid; F, flocculation.

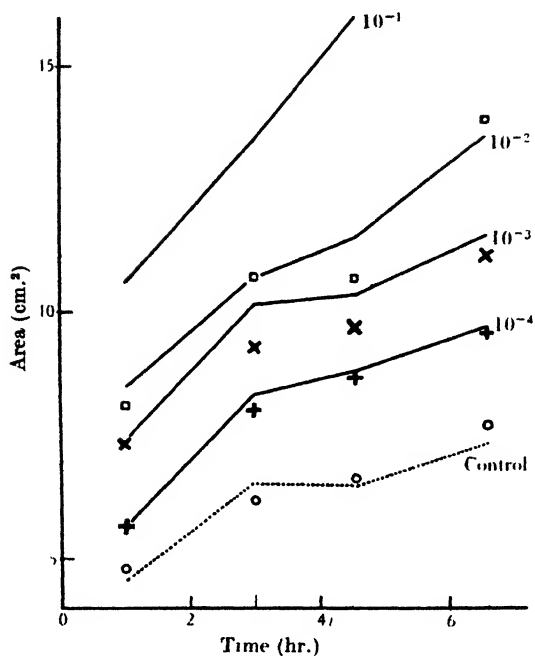


Fig. 5. Fractionation with  $(\text{NH}_4)_2\text{SO}_4$ . Activity of precipitates.

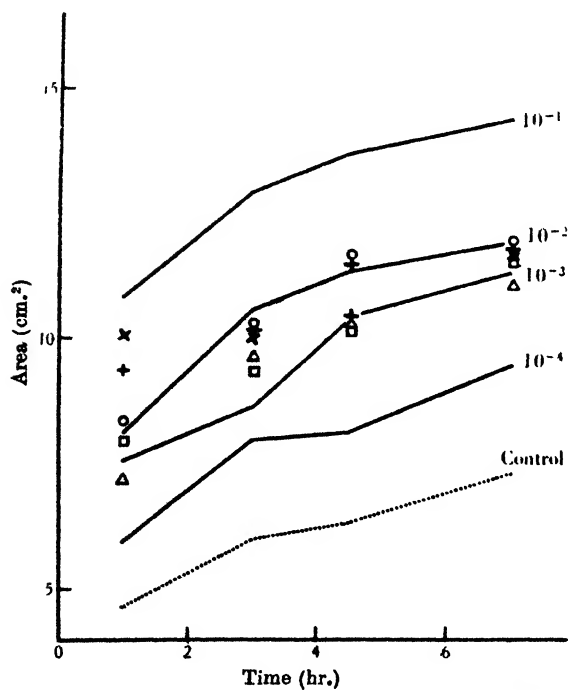


Fig. 6. Fractionation with  $(\text{NH}_4)_2\text{SO}_4$ . Activity of supernatants.  
 ○ 20%; + 33%; × 50%; □ 75%; △ 90%

*Adsorption of crude testicular extracts on aluminium oxide*

250 g. dry testicle powder were extracted (mechanical stirring for 1 hr.) three times with 4 l. of water. The insoluble material was separated each time

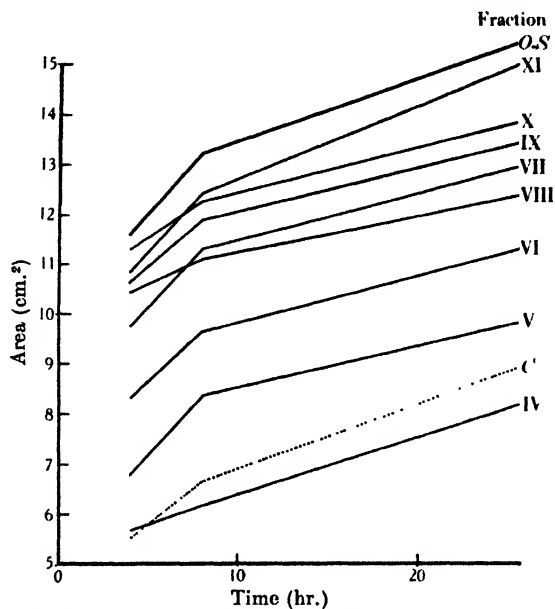


Fig. 7. Adsorption of crude testicular extract on aluminium oxide

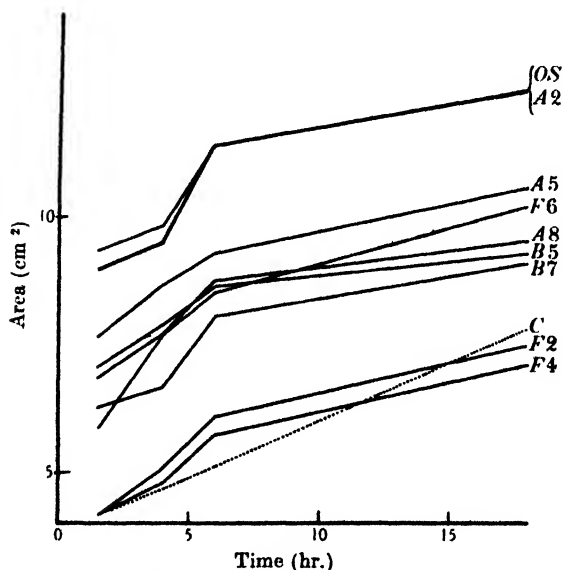


Fig. 8. Adsorption and elution of crude testicular extracts.

by decantation, and the supernatants mixed. To these 100 ml. *N*/10 acetic acid were added and the precipitate allowed to sediment in the cold room. The clear

supernatant was passed through a column ( $10 \times 4.5$  cm.) of aluminium oxide (Merck) (100 g.) previously wetted with water. The receiver was changed every 250 ml. (30 min.). After the first four fractions were collected the vacuum was increased and the receiver changed every 500 ml. (20 min.). Two yellowish bands were observed, the first reaching the bottom of the column when 3 l. of solution had passed through. In the table below some of the properties of the resulting fractions are recorded. Tenfold dilutions (in isotonic haemoglobin solution) of fractions iv–xi were injected into the back of three rabbits. The spread of haemoglobin through the skin is recorded in Fig. 7. Fraction vi giving tests for protein has not the full activity of the original solution (OS) while fraction ix, although it does not give a precipitate with lead acetate, has almost the same activity as the original solution.

Fraction no.	OS	i	ii	iii	iv	v	vi	vii	viii	ix	x	xi
Vol. of fraction in ml.	.	250	250	250	250	550	500	400	500	475	500	600
Vol. of liquid through column in l.	.	0.25	0.5	0.75	1	1.5	2	2.5	3	3.5	4	4.5
Precipitation by trichloroacetic acid	+	—	—	—	—	—	+	+	+	+	+	+
Precipitation by neutral lead acetate	+	+	±	±	±	±	+	+	±	—	+	+
Heat-coagulation	+	—	—	—	—	+	+	+	+	+	+	+
Buuret test	+	—	—	—	±	+	+	+	+	+	+	+

In another experiment a similar column of aluminium oxide (Hopkins and Williams pure anhydrous) was prepared. 300 ml. of the testicular extract used in the previous experiment (2.63 mg. N/5 ml.) were filtered through it, changing the receiver every 50 ml. The column was then washed with 400 ml. *M*:500 acetic acid and subsequently with 1%  $\text{NaHCO}_3$  (400 ml.). The behaviour towards trichloroacetic acid and the N content of some of the fractions collected are shown in the following table.

		Filtrates (F)							
Fractions of 50 ml.		1	2	3	4	5	6		
Trichloroacetic acid		-	-	-	+	+	+		
mg. N/5 ml.	.	.	0.014	.	0.45	.	0.94		
		Acetic acid washings (A)							
Fractions of 50 ml.		1	2	3	4	5	6	7	8
Trichloroacetic acid	+	+	+	+	+	+	+	±	±
mg. N/5 ml.	.	.	2.48	.	0.384	.	.	.	0.128
		Bicarbonate washings (B)							
Fractions of 50 ml.		1	2	3	4	5	6	7	8
Trichloroacetic acid	±	?	?	+	+	±	±	±	?
mg. N/5 ml.	.	.	.	0.331	.	.	.	0.069	.

The area of skin over which haemoglobin is induced to spread by injection of 1:10 dilutions (in isotonic haemoglobin solution) of some of these fractions is shown in Fig. 8. After most of the active materials have been eluted with acid, substantial amounts of inactive protein are eluted from the column by  $\text{NaHCO}_3$ .

## SUMMARY

1. Comparison of testicular diffusing preparations with kallikrein indicate that, contrary to the statement of Christensen [1938], the two materials are quite unlike one another in their effects on the rate of spread of injected fluids through rabbit skin.

2. Rattlesnake venom, filtrates of *Clostridium Welchii* and *Vibrio septique*, staphylococcal toxoid, leech extracts and diazobenzenesulphonic acid-serum globulin all resemble the testicular diffusing factor in producing a very rapid increase in the rate of spread of fluids through skin.

3. Purification of the testicular diffusing factor by means of fractional precipitation with ammonium sulphate and dialysis is described. This method yields products of similar activity to those previously described [Madinaveitia, 1938].

4. The main bulk of evidence indicates that the active material has protein properties and that its biological action, which does not depend on circulation, is not due to a surface tension effect.

The author wishes to express his thanks to Prof. A. R. Todd and Dr D. McClean for their continued interest and encouragement. He is also grateful for the hospitality of the Lister Institute where the early part of this work was carried out. Gifts of bacterial filtrates from Dr D. McClean and of rattlesnake venom from Dr F. Duran-Reynals are gratefully acknowledged.

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# XLV. THE MECHANISM OF THE FORMATION OF ORGANIC ACIDS BY MOULD FUNGI

## III. THE INFLUENCE OF IODOACETATE AND OF FLUORIDE ON THE FORMATION OF ACIDS

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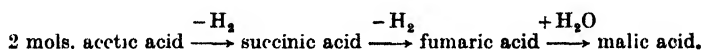
*(Received 31 January 1939)*

THE action of *A. niger* in the presence of sodium iodoacetate [Johnson *et al.* 1937] has now been studied with sucrose as the substrate and the experiments have yielded results similar to those found with glucose. Thus  $M/450$  iodoacetate inhibited anaerobic formation of ethyl alcohol by six different strains of *A. niger* which were able in the presence of  $M/400$  iodoacetate to produce both citric and oxalic acids in quantities sufficient for quantitative estimation. Further, although  $M/400$  iodoacetate restricted the average quantity of sugar metabolized in 11 days to about 30%, little difference was noted in the yield of gluconic acid in g. per 100 ml. culture liquid, since the yield of the latter acid expressed as a percentage of the weight of sugar consumed was raised from an average normal value of 13% to a value of 45% in the presence of  $M/400$  iodoacetate (Table I).

Also, experiments conducted simultaneously in the presence and in the absence of iodoacetate showed that the latter substance at concentrations of  $M/1000$  and  $M/500$  caused a large rise in the weight of citric acid and a considerable fall in the weight of gluconic acid produced in unit volume of the culture medium (Table II). After incubation for 20 days in presence of  $M/500$  iodoacetate the sugar metabolized was found to be 80% of the quantity present initially, as compared with a consumption of 66.75% in absence of iodoacetate.

Experiments were also undertaken to ascertain the behaviour of *A. niger* towards sucrose in the presence of NaF. Using strain T1 it was necessary to employ NaF at a concentration of  $M/100$  in order completely to suppress the formation of citric acid, whereas under anaerobic conditions the formation of ethyl alcohol was inhibited in the presence of  $M/200$  NaF. In further experiments with strain T1 conducted aerobically in the presence of NaF at concentrations from  $M/200$  to  $M/400$  it was found in every case that in 13 days the total acid produced was less than in the "controls" without NaF. The amounts of citric acid formed were particularly low in comparison with those produced in the "control" flasks but, on the other hand, the formation of gluconic acid, which was negligible in the "control" flasks, was strongly stimulated by all concentrations of NaF and reached a maximum when the latter was  $M/250$  (Table III). The effects of NaF on acid formation by *A. niger* are similar, in some respects, therefore, to those exercised by iodoacetic acid. Bernhauer [1928] has reported that in the presence of certain concentrations of the commercial germicide "Uspulin" the action of *A. niger* on sugar results in largely increased production of gluconic acid with simultaneous suppression of the formation of citric acid.

The increase of gluconic acid formation in cases such as these may be explained in two ways. The poison may selectively cripple the enzyme system or systems responsible for the formation of citric acid whilst leaving undamaged, or even stimulating, the glucose oxidase which gives rise to gluconic acid. This would leave the glucose oxidase free to transform into gluconic acid the sugar which normally would be converted into citric acid. Alternatively, it is possible that the poison cripples the enzyme system employed by the mould for the conversion of gluconic acid into citric acid. If such is the case the experimental results in question are evidence in favour of the view that gluconic acid is an intermediate in the formation of some, at least, of the citric acid produced from sucrose. Chrzęszcz & Tiukow [1930] suggested that the first phase of citric acid formation from sugar under the influence of mould fungi consisted of the processes of alcoholic fermentation, and that this phase was succeeded by one in which acetic acid (formed from acetaldehyde or ethyl alcohol) gave rise to 4-carbon dicarboxylic acids in the following sequence:



The authors presumed that citric acid was then produced by a final dehydrogenation whereby 1 mol. of malic acid was condensed with 1 mol. of acetic acid. Whilst the subsequent work of a number of investigators [Butkewitsch & Gajevskaya, 1935; Wells *et al.* 1936; Clutterbuck, 1936; Walker, 1936] has proved that alcoholic fermentation cannot be an initial phase in citric acid formation, the experiments of Chrzęszcz & Tiukow [1930], of Chrzęszcz *et al.* [1932] and of Bernhauer *et al.* [1934] have fully substantiated the formation of succinic, fumaric, malic and citric acids in cultures of various moulds in contact with salts of acetic acid or with very dilute ethyl alcohol. Hence, if it could be shown that mould fungi can transform 1 mol. hexose into 3 mol. acetic acid or into 1 mol. acetic acid together with 1 mol. of any one of the above 4-carbon acids, it would then be justifiable to regard the later reactions immediately preceding the formation of citric acid as consisting of dehydrogenations of the type suggested by Chrzęszcz & Tiukow. An explanation on these lines would, moreover, approximate very closely to the hypothesis of Raistrick & Clark [1919], who viewed the initial processes of citric acid formation as consisting of dehydration and oxidation of the hexose molecule followed by fission into 1 mol. of acetic acid and 1 mol. of oxaloacetic acid, the two latter substances constituting the immediate precursors of the citric acid to which, by an aldol condensation, they were presumed to give rise. Views similar to those of Raistrick & Clark were also expressed later by Virtanen & Pulkki [1930]. Whilst these assumptions are quite in harmony with the known properties of the several substances involved, neither acetic acid nor oxaloacetic acid has yet been isolated from cultures of *A. niger* on sugar. According to Bernhauer *et al.* [1932] small quantities of malic acid may sometimes be detected in cultures of this mould on sugar solutions, a statement which we can confirm from our own experiments, and possibly this malic acid arises by reduction of oxaloacetic acid. It is equally possible, however, that such malic acid is derived from succinic acid, and this view leads to consideration of the possibility mentioned above, namely, that fission of the sugar molecule under the influence of the mould may give rise to the production of equimolecular quantities of acetic and succinic acids. Support for the belief that a non-phosphorylated glucose molecule can, by reactions involving fission, give rise to succinic acid, is found in the phenomena observed by Virtanen [1925] when investigating cell-free propionic acid fermen-

tation, cf. Virtanen & Simola [1927]. Moreover, Scheffer [1928], working with Kluyver, obtained quantitative data in favour of the conception that in the fermentation of glucose by *B. coli* some of the sugar is split into 4-carbon and 2-carbon compounds. These observations led one of us to suggest [Johnson, 1934] that formation of citric acid may take place through an initial conversion of a molecule of hexose into 1 mol. acetic acid and 1 mol. succinic acid, and that these two acids would then give rise to the final product in accordance with the scheme of Chrzęszcz & Tiukow or with one of the closely related schemes suggested by Bernhauer *et al.* [1934]. Alternatively and with equal reason the succinic acid could be assumed to undergo conversion into oxaloacetic acid when the final condensation would be that postulated by Raistrick & Clark.

Gudlet [1936], from consideration of Virtanen's work, has also arrived at the same opinion and has suggested that the conversion of sugar into acetaldehyde and succinic acid, without loss of CO<sub>2</sub> by decarboxylation, is a necessary step in the production of citric acid by moulds.

Facts which have been co-ordinated by Kluyver [1935] would seem to provide a reasonable explanation of the mechanism of the formation of both acetic and succinic acids from glucose by microbiological agency. He has brought forward the view that the formation of these acids from glucose by *B. coli* occurs through an initial fission of the hexose to ethylene glycol and tartaric acid dialdehyde, followed by transformation of the former into acetaldehyde and of the latter into succinic acid. Further, the well known formation of equimolecular quantities of acetic and lactic acids from xylose by the action of *Lactobacillus pentosus* is a conversion which is readily understandable if considered as completely analogous to the case of the action of *B. coli* on glucose, for the xylose may be presumed to yield glycollic aldehyde and glyceric aldehyde which are then transformed into acetic acid and lactic acid, respectively.

Consideration of the citric acid problem from this standpoint has led us to devise further experiments to gain information respecting the processes involved in the production of this acid from sugar. If succinic acid is an intermediate it follows that any agent which serves to inhibit its oxidation might also be expected to exercise an inhibitory effect on the production of citric acid in cultures of *A. niger* on sugar. Accordingly, in view of the fact that we had already shown that *M*/500 iodoacetate does not interfere with the production of citric acid from sugar, we next examined the behaviour of citric acid-forming moulds towards Na acetate in the presence of iodoacetate, and found that at *M*/500 concentration of the latter poison the synthesis of both malate and citrate from acetate by two species of *Penicillium* was not prevented and, in agreement with this result, we also made the observation that *M*/500 iodoacetate did not interfere with the conversion of succinic acid into *l*-malic acid under the influence of *A. niger* (strain N1). In the presence of *M*/450 iodoacetate *l*-malic acid was still produced, though in diminished yield, from succinic acid. According to Quastel & Whetham [1924] NaF strongly inhibits succinodehydrogenase. Since we had found that NaF at concentration *M*/150 allowed the formation by *A. niger* of a little citric acid from cane sugar, whereas at concentration *M*/100 no citric acid was obtained, the effects of NaF were tried in cultures of *A. niger* on succinic acid. It was found that whereas malic acid was still produced (though only in traces) when the concentration of fluoride was raised to *M*/150, at *M*/100 the formation of malic acid could no longer be demonstrated. It appears, therefore, that the respective concentrations of iodoacetate and NaF which were found to be just sufficient to prevent the mycological conversion of sugar into citric acid are also the concentrations which are necessary to prevent



the conversion of succinate (or acetate) into malate or citrate. Hence, none of the evidence provided by our experimental results disagrees with the suggestions made by one of us [Johnson, 1934] of a possible mechanism of citrate formation from sugar, *via* succinate plus acetate.

#### EXPERIMENTAL

The moulds employed were six pure strains of *A. niger*, designated N1, N2, B7, B12, B16 and T1, respectively, and cultures of *Penicillium citroenum* and of *P. Johannoli*. The two last named are citric acid-producing species which were kindly placed at our disposal by Prof. T. Chrzyszcz of Poznań. For experimental purposes they were cultivated in most cases on a sterilized medium containing 0.2%  $\text{NH}_4\text{NO}_3$ ; 0.1%  $\text{KH}_2\text{PO}_4$ ; 0.05%  $\text{MgSO}_4$ , 7H<sub>2</sub>O and glucose or sucrose, 10 or 12.5%.

#### *Action of A. niger on sucrose in presence of M/400 Na iodoacetate*

Six strains of *A. niger* were grown at 30° from spores sown on the nutrient salt medium containing 10% of sucrose in 24 Erlenmeyer flasks of 1500 ml. capacity, each flask containing 300 ml. liquid. After 5 days the sugar solutions were poured away, the mycelia were washed as described [Johnson *et al.* 1937] and a fresh medium consisting of 10% sucrose containing M/400 Na iodoacetate was introduced under the mycelia in 12 of the flasks. The other 12 flasks were recharged with 10% sucrose without iodoacetate and were used as controls. All the 24 flasks were then returned to the incubator for a further period of 11 days. The analytical figures are shown in Table I.

Table I. *Iodoacetic acid (as Na salt) at concentration M/400 in 10% cane sugar solution*

Strain of <i>A. niger</i>	Glucose consumed, % of initial wt.	Acidity, ml. 0.1N NaOH to neutralize 100 ml. medium	Acid produced, expressed as % by wt. of the sugar consumed		
			Citric acid	Oxalic acid	Gluconic acid
T 1	13	36.3	5.1	0.7	36.3
N 1	14	45.1	3.0	1.7	46.5
N 2	11	30.8	3.3	1.1	40.3
B 12	11	31.9	2.6	1.7	41.3
B 7	14	49.5	3.2	2.1	50.4
B 16	15	59.4	5.5	1.4	55.1
"Controls", in absence of iodoacetate					
T 1	43.5	110	10.7	—	17.0
N 1	66.6	271	9.7	9.1	17.4
N 2	55.6	150	12.6	2.2	5.9
B 12	62.5	213	13.6	1.5	19.3
B 7	56.0	147	11.0	0.6	15.0
B 16	47.8	117	14.2	0.4	2.9

In order to establish beyond doubt the identities of the three acids (presumed to be citric, oxalic and gluconic, respectively) produced in presence of M/400 iodoacetate (Table I), they were converted into derivatives. From the two first named, the *p*-nitrobenzyl esters were prepared; they melted at 102 and 204°, respectively, alone or in admixture with authentic specimens. The Ca gluconate was purified [cf. Butkewitsch, 1924] and dried at 100–110°. Found: CaO, 12.9%. Calc.: CaO, 13.02%. The phenylhydrazide of the acid was prepared and sintered

at 195°, M.P. (decomp.) c. 200° [cf. Butkewitsch, 1924; Fischer & Passmore, 1889]. Found: C, 50.2; H, 6.5; N, 10.0%.  $C_{12}H_{18}O_6N_2$  requires C, 50.35; H, 6.3; N, 9.8%.

*Action of A. niger on sucrose in presence of Na iodoacetate M/1000 and M/500*

The mould T1 was cultivated in 20 flasks of 350 ml. capacity each charged with 100 ml. 10% sucrose saline medium. After growing for 4 days at 30° the mycelia were washed and the medium in 10 flasks was replaced by 10% sucrose and in the other 10 flasks by 10% sucrose together with M/1000 Na iodoacetate. This experiment was repeated with another series of 20 flasks, in which case M/500 iodoacetate was used. For the analyses, samples withdrawn from each of the 10 flasks which formed a series were mixed; thus the figures represent the averages of ten values in each case. The data are shown in Table II.

Table II. *A. niger*, T1, incubated at 30° on 10% sucrose solution in presence and in absence of M/1000 and M/500 Na iodoacetate

Time in days	Acidity, ml. 0.1N NaOH to neutralize 100 ml. medium	Acidity expressed as mg. acid per 100 ml. medium			Acidity, ml. 0.1N NaOH to neutralize 100 ml. medium	Acidity expressed as mg. acid per 100 ml. medium		
		Citric acid	Oxalic acid	Gluconic acid		Citric acid	Oxalic acid	Gluconic acid
In presence of M/1000 iodoacetate								
3	30	+	—	—	62	+	—	—
6	58	+	—	—	95	39	—	1744
8	64	155	+	842	116	78	22	2000
10	72	227	+	725	125	115	46	2000
13	84	266	3	842	158	143	89	2646
15	96	305	5	960	166	127	115	2606
17	106	337	5	1038	174	95	156	2783
22	144	470	202	1352	204	82	237	3234
In presence of M/500 iodoacetate								
6	58	216	—	666	86	93	—	1460
8	66	237	—	568	98	96	63	1489
10	74	258	Trace	666	110	98	73	1705
13	96	358	103	686	134	102	101	2097
15	108	340	155	744	158	132	125	2430
17	118	335	193	862	169	145	145	2556
20*	136	238	345	1192	152	102	163	2313

\* On the 20th day sugar estimations showed that in the flasks containing M/500 iodoacetate the sugar consumption had amounted, on the average, to 80% of the quantity initially present, whilst in absence of iodoacetate 66.75% of the sugar had been consumed.

*Action of A. niger on sucrose in the presence of NaF*

The mould T1 was cultivated on a 10% sucrose medium as in the previous experiments, and on the 5th day from the time of inoculation this medium was poured away, the mycelia were washed, and fresh 10% cane sugar solutions, containing different concentrations of NaF, were introduced under the mycelia in the several flasks. The culture media were examined after further incubation at 30° for 6 days and 13 days, respectively. In Table III the analytical data obtained on the 13th day are given. Oxalic acid was not detected in any of the flasks, and no citric acid could be detected in presence of M/100 NaF.

Table III. *A. niger*, *T*1, incubated at 30° for 13 days on 10% sucrose solution in the presence of NaF

Conc. of NaF	Sugar consumed, % of initial wt.	Acidity, ml. 0.1N NaOH to neutralize 100 ml. medium	Citric acid: (a) mg. per 100 ml. medium; (b) % of wt. of glucose consumed		Gluconic acid: (a) mg. per 100 ml. medium; (b) % of wt. of glucose consumed	
			(a)	(b)	(a)	(b)
N/150	28	36.3	20	0.7	664	23.6
N/200	28.5	38.5	36	1.3	650	22.9
N/250	34	55.0	62	1.8	900	27.0
N/300	32	48.0	94	2.9	700	24.9
N/400	23	22.0	47	2.0	294	12.8
Control (no fluoride)	45	68.2	422	9.4	44	1.0

*The action of Penicillium citrogenum and of P. Johannili on Na acetate in the presence of M/500 Na iodoacetate*

These two *Penicillium* species were chosen in preference to *A. niger* for this particular experiment since they had been found by Chrząszcz & Tiukow to be particularly suitable for demonstrating the mycological production of citric acid from acetic acid. Moreover, previous experiments with *P. citrogenum* had shown that when it was placed on a 12.5% glucose medium production of citric acid was considerably curtailed in presence of *M/500* iodoacetate.

Cultures of the two moulds were grown in 24 boiling tubes (12 tubes for each species) from spores sown on a medium consisting of sucrose (5%) in nutrient inorganic salt solution. After incubation for 4 days at 25° the mycelia were transferred to 24 conical flasks (1500 ml.) each containing 330 ml. of the same medium and incubation at 25° was conducted for 6 days, after which the mycelial mats were washed thoroughly with sterile water and allowed to stand overnight over water. After this treatment they no longer diffused citric acid into the water. The mycelia were then floated on a sterile aqueous solution of Na acetate (5.6% equivalent to 2.5% acetic acid) containing also *M/500* Na iodoacetate. The metabolism solutions were collected for examination after the mycelia had been in contact with the acetate medium for 8 days at 25°.

(1) *P. citrogenum* cultures. The mixed culture fluid from the 12 flasks was filtered and evaporated to one-third of its volume after which sat. Pb acetate was added to it in slight excess and the mixture was allowed to stand for 3 hr. The white precipitate which had formed was filtered and washed at the pump until the washings were free from Pb. The filtrate (A) was preserved for examination. The washed precipitate was suspended in 1750 ml. water, 600 ml. 2% acetic acid were added and the whole was warmed for 2 hr. on a steam bath and then filtered. The undissolved solid matter was well washed and the washings were added to the filtrate (B) which was preserved for examination. The washed solid (C) was suspended in water and decomposed by H<sub>2</sub>S. The PbS was removed by filtration and the filtrate was evaporated to one-third of its volume. In this liquid were found the largest proportions of acidic products and these were dealt with as follows. Qualitative tests having shown the presence of citric and oxalic acids, these were separated through their Ca salts in the usual manner. A portion of the fraction which was presumed to be Ca citrate after repeated crystallization from hot water and drying at 130° gave 0.2451 g. pure Ca salt. Found: CaO, 33.9%. Calc. for anhydrous tricalcium citrate: CaO, 33.74%. A further portion (1.5 g.) of the same material was treated with the calc. H<sub>2</sub>SO<sub>4</sub> and, after removal of the CaSO<sub>4</sub>, the solution was

evaporated to one-half of its volume, neutralized with 0.5*N* alcoholic KOH and finally evaporated to dryness. To the solution of the K salt in 10 ml. water were added 30 ml. alcohol and 2 g. *p*-nitrobenzyl bromide. The whole was heated under reflux for 2 hr. after which, on cooling, the product separated and was collected and recrystallized from aqueous alcohol. The purified material had m.p. 102° alone or in admixture with an authentic and pure specimen of the tri-*p*-nitrobenzyl ester of citric acid.

The Ca salt obtained from the fraction presumed to contain oxalic acid was purified in the usual manner and analysed. Found: Ca, 38.11%. Calc. for  $C_2O_4Ca, H_2O$ : Ca, 38.36%. A small quantity of the acid was converted into its *p*-nitrobenzyl ester which, after purification, had m.p. 204° alone or in admixture with an authentic specimen of the di-*p*-nitrobenzyl ester of oxalic acid.

Filtrate (A) was evaporated to one-half of its volume and this concentrate was mixed with 3 vol. of alcohol, but only a faint cloudiness appeared and no substance could be identified. Filtrate (B) was treated with  $H_2S$ , the precipitate of PbS removed and the filtrate evaporated, when a small quantity of sticky solid remained. This was freed from the last traces of water and acetic acid by addition of a mixture of benzene and alcohol and subsequent evaporation *in vacuo*. About 0.5 g. solid material remained which gave a strong positive reaction for malic acid when submitted to Denigès' Hg acetate test. This was confirmed by conversion of the acid into acetaldehyde which in turn was characterized by condensation with dimethyldihydroresorcinol to yield aldomedon, m.p. 142°.

(2) *P. Johannioli* cultures. The metabolism solution from these cultures was treated in a similar manner. Citric acid and oxalic acid were both isolated and characterized as their respective *p*-nitrobenzyl esters, but the quantities of these acids were less than those obtained in the case of *P. citrogenum*.

*The action of A. niger on NaH succinate in the presence  
of Na iodoacetate*

Seventeen conical flasks (1000 ml.) were each charged with 250 ml. sucrose (5%) in the nutrient salt medium of Wells *et al.* [1936]. After sterilization the solutions were inoculated with cultures of *A. niger*, strain N1, previously developed from spores in boiling-tubes containing the same medium. After incubation for 5 days at 30° the culture fluid was poured away and the mycelial mats were washed with sterile water and allowed to stand for 6 hr. over fresh sterile water. The latter was then poured away and seven of the flasks were each charged with 250 ml. of a sterile 4% aqueous solution of NaH succinate containing *M*/500 Na iodoacetate. Seven other flasks were treated similarly except that in these the concentration of iodoacetate was *M*/450. The remaining three flasks contained succinate without iodoacetate as controls. All the flasks were returned to the incubator at 30° and from time to time samples were withdrawn and tested for the presence of malic acid by Denigès' Hg acetate solution. Positive tests were obtained after 7 days, and after 14 days the solutions were combined into two lots and examined. Since the colour in both cases was too dark to permit direct polarimetric examination, aliquots (100 ml.) of each were made alkaline with strong  $NH_3$  and treated with excess Pb acetate. After the addition of 50 ml. alcohol each solution was allowed to stand for 48 hr. The precipitates which had settled were then filtered and after washing with 30% alcohol they were ground with water to a fine suspension and treated with  $H_2S$ . The PbS was removed, the clear solutions were boiled to expel  $H_2S$  and

their rotations were measured in 1 dm. tubes in the presence of ammonium molybdate [Williamson, 1918] with the following results: sample from *M*/500 iodoacetate flasks,  $+1.6^{\circ}$  (Ventzke scale); sample from *M*/450 iodoacetate flasks,  $+0.6^{\circ}$ ; sample from control iodoacetate flasks,  $+0.85^{\circ}$ . Since molybdate reverses the sign of rotation, these results represent *laevo* rotations caused by *l*-malic acid. A preliminary test had shown that *M*/450 iodoacetate did not affect the accuracy of Williamson's method. Since the separation of Pb salts was not quantitative, the values for the solutions incubated in presence of iodoacetate cannot be compared accurately with the "control".

*The action of A. niger on NaH succinate in the presence of NaF*

Preliminary experiments indicated that spores of *A. niger* (strain N1) would germinate in sugar solutions containing *M*/150 NaF but not in *M*/100. To each of a series of conical flasks (1000 ml.) containing 250 ml. of a 4 % aqueous solution of NaH succinate, a different quantity of NaF was added and three flasks were left as controls without NaF. All were inoculated with spores of *A. niger* (strain N1) and were incubated at  $30^{\circ}$ . The mould grew freely in all the flasks and after 14 days Denigès' test for malic acid was positive in all cases, but the amounts of malic acid detected, including those found in the control flasks, were very small. After 28 days a mixed sample from the controls was treated with ammonium molybdate for polarimetry in a 2 dm. tube, when the rotation was found to be  $+0.45^{\circ}$  (Ventzke scale) corresponding to a 0.015 % solution of *l*-malic acid, while the culture liquid containing *M*/150 NaF on similar treatment showed  $+0.3^{\circ}$ , corresponding to 0.01 % *l*-malic acid. A preliminary test had shown that *M*/150 NaF did not interfere with the polarimetric estimation of malic acid by this procedure.

SUMMARY

1. The effects of *M*/400 Na iodoacetate on the behaviour of *A. niger* (6 strains) in contact for 11 days at  $30^{\circ}$  with 10 % sucrose solution, as shown by comparison with cultures incubated in absence of iodoacetate, resulted in (a) a restriction in the total acid formation; (b) a restriction in the quantity of sugar used to about 30 % of that normally metabolized; (c) a decrease in the yield of citric acid expressed as a percentage of the sugar consumed; and (d) an increase in the yield of gluconic acid expressed as a percentage of the sugar consumed.

2. Iodoacetic acid (*M*/1000 or *M*/500 as Na salt) in 10 % sucrose solution in contact with the fully developed mycelium of *A. niger* (strain T1) at  $30^{\circ}$  caused in a given time: (a) a diminished acid formation; (b) a large increase in the weight of citric acid formed; and (c) a large decrease in the weight of gluconic acid formed, as compared with a control experiment made in absence of iodoacetate.

3. NaF when added at concentrations from *M*/150 to *M*/400 to cultures of *A. niger* on cane sugar depressed the consumption of the sugar, the development of titratable acidity and the formation of citric acid. Spores of *A. niger* did not germinate in a nutrient salt-sugar medium containing *M*/100 NaF, and the same concentration of this poison was found to suppress completely the formation of citric acid by mycelia which had been grown normally before being brought into contact with fluoride.

4. Na iodoacetate (*M*/500) did not prevent the formation of malic and citric acids in cultures of *Penicillium citrogenum* and *P. Johannioli* on a Na acetate solution.

5. Na iodoacetate,  $M/500$  or  $M/450$ , did not prevent the formation of *l*-malic acid from succinic acid by *A. niger* (strain N1).

6. NaF ( $M/150$ ) did not prevent the formation of *l*-malic acid from succinic acid by *A. niger* (strain N1).

The results summarized under headings 4, 5 and 6, when considered in conjunction with the other results described in this communication and in Part II [Johnson *et al.* 1937], provide no evidence against the view that succinic and acetic acids are intermediates in the mycological conversion of sugar into citric acid.

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# XLVI. THE ACTION OF THE DIPROPIONATE AND BENZOATE-BUTYRATE OF OESTRADIOL ON OVARIECTOMIZED RATS

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*(Received 27 January 1939)*

MIESCHER *et al.* [1938] have prepared several aliphatic di-esters of oestradiol which they have carefully tested, using the methods of vaginal smear and uterine growth. They gave subcutaneous injections of the di-esters to immature normal and ovariectomized rats, the total dose being usually 50  $\mu$ g., given in equal parts on two consecutive days, and found that, as compared with oestradiol, the di-esters have an extremely intense and prolonged effect. The di-esters are therefore important both for experimental and clinical purposes.

In the present paper are given the results of ten experiments (Tables I and II, groups III–VIII, XII–XV), in which the esters were injected for a period of 3 weeks, and of one experiment (Table I, group IX) for 19 weeks. The dipropionate and the 3-benzoate-17-*n*-butyrate were chosen as being typical representatives of the series of aliphatic and mixed aliphatic-aromatic di-esters.

Two esters, the effects of which last for very different lengths of time, were deliberately chosen: a long duration of effect would appear to be most advantageous for clinical use, but it is necessary to consider also the disadvantage of being unable to stop the effects of the injection if this should be required. In this respect there might even be danger, with a hormone which produces not only physiological but also pathological effects, as is the case with all oestrogens. According to Miescher *et al.* (p. 731, fig. 7) the maximal growth of the uterus with the dipropionate was obtained on the 12th day, after which period the effect started to decline, while with the benzoate-butyrate the maximal growth was still present and had not begun to decline on the 50th day. Robson *et al.* [1938], injecting a total dose of 50  $\mu$ g. of the benzoate-butyrate into mice, found that the effect of the hormone lasted more than 120 days, but that after 95 days the degree of its action was already halved.

## *Technique*

The hormones were supplied by Messrs Ciba Ltd. Our technique of preparation was as usual: the hormones were first dissolved in alcohol, then mixed with warmed sesame oil and the alcohol distilled off *in vacuo* at a temperature rising slowly from 70 to 85°. The hormonal solution was then put into ampoules, sealed and sterilized for 3 days at 100°. The ampoules were kept in the cold store until needed. While oestradiol benzoate-butyrate was not affected by this method of solution, the dipropionate appeared to be weakened, since after keeping for a few months, even in the cold store, its effect was less strong. Dr Miescher has suggested (personal communication) that with our method of solution some of the ester might possibly be decomposed with liberation of pure oestradiol, and that shaking the dipropionate in oil warmed up to 70° without any additions

gives the most stable solution. However, since we discarded all experiments in which we noticed a weaker effect of the dipropionate and used fresh solutions, the duration of the effect in our experiments has been similar to that in the experiments of Miescher *et al.*

The rats used were ovariectomized on the 22–26th day of age. The hormones were injected as follows: the dipropionate was injected for three consecutive weeks in weekly doses of 18 and 90  $\mu\text{g.}$  (6 and 30  $\mu\text{g.}$  respectively three times a week, Table II, groups XII–XV); the smallest dose 18  $\mu\text{g.}$  of the benzoate-butyrate (Table I, groups III–V) was injected similarly, and the dose of 90  $\mu\text{g.}$  (groups VI–VIII) was given once a week for three consecutive weeks; in group IX, the weekly dose of 300  $\mu\text{g.}$  was given (100  $\mu\text{g.}$  three times a week) for 19 weeks.

In order to ascertain the duration of the effect after the last injection the rats in groups IV, V, VII, VIII, XIII and XV ("10–106 days rest") were left without injection for the requisite number of days before killing. In order that in each experiment all the rats might be killed on the same day, the injections into the rats of groups "10–106 days rest" were started the respective number of days earlier than the injections into the rats which were killed on the next day after the last injection (groups III, VI, IX, XII and XIV).

In this way the final ages of comparable groups were the same. We have not noticed any definite effect through starting the injections at an earlier age in some groups than in others.

Other details of our technique were the same as in our previous experiments. The total number of rats used was 99. The average age of the rats in Table I was 107–176 days (col. X), and in Table II 88 days.

Table I. *Effects of oestradiol benzoate-butyrate on actual weights of organs of ovariectomized rats*

Organs	Weekly doses of oestradiol benzoate-butyrate									
	Control rats		Injected for 3 weeks						Injected for 19 weeks	Average final age in days
	Normal	Ovariectomized	18 $\mu\text{g.}$	18 $\mu\text{g.}$ + 30 days rest	18 $\mu\text{g.}$ + 52 days rest	90 $\mu\text{g.}$	90 $\mu\text{g.}$ + 30 days rest	90 $\mu\text{g.}$ + 106 days rest	300 $\mu\text{g.}$	
	I	II	III	IV	V	VI	VII	VIII	IX	X
Uterus (horns + cervix, mg.)	411	36	228	276	271	248	290	—	—	107
	549	43	—	—	—	—	—	292	536	176
Vagina with clitoris (mg.)	232	175	314	311	353	258	328	—	—	107
	326	189	—	—	—	—	—	365	409	176
Preputial glands (mg.)	97	58	73	67	67	78	81	76	58	107–76
Hypophysis (mg.)	14.7	12.5	18.8	23.2	20.2	30.7	42.2	32.0	76.0	107–76
Thymus (mg.)	283	440	397	385	313	257	277	—	—	107
	213	221	—	—	—	—	—	197	110	176
Liver (g.)	8.34	9.08	8.69	11.02	10.67	9.36	9.41	8.22	8.45	107–76
Kidneys (g.)	1.53	1.61	1.82	2.02	1.83	1.99	1.86	—	—	107
	1.87	1.79	—	—	—	—	—	1.92	1.82	176
Abdominal fat (g.)	21	20	10	15	17	12	12	16	7	107–76
Final body wt. (g.)	244	308	245	288	283	247	224	—	—	107
	280	322	—	—	—	—	—	266	198	176
No. of rats in the group	10	9	4	6	3	7	7	4	4	107–76

*Note.* In the control groups I and II two average weights are given only for those organs of which the weight varies considerably according to the age (column X). In group I the vaginal weight of rats aged 107 days is given without clitoris.



Table II. *Effects of oestradiol dipropionate on actual weights of organs of ovariectomized rats*

Organs	Weekly dose of oestradiol dipropionate				
	Control sprayed rats XI	18 $\mu$ g. XII	18 $\mu$ g. + 10 days rest XIII	90 $\mu$ g. XIV	90 $\mu$ g. + 10 days rest XV
Uterus (horns + cervix, mg.)	38	249	210	252	232
Vagina with clitoris (mg.)	170	285	313	268	281
Preputial glands (mg.)	49	74	63	67	65
Hypophysis (mg.)	10.9	22.1	21.3	35.5	32.4
Thymus (mg.)	467	299	313	218	223
Liver (g.)	8.56	9.00	11.35	9.38	9.32
Kidneys (g.)	1.57	1.84	1.97	2.10	1.82
Abdominal fat (g.)	17	9	13	7	11
Final body weight (g.)	276	223	273	223	211
Gain in body weight (g.)	76	21	39	28	27
No. of rats in the group	10	12	8	7	8

*Effect on sex organs*

The maximum effect, as studied by weight of the organs, was slightly greater with the benzoate-butyrate than with the dipropionate. We have not studied the duration of the effect of the dipropionate for a longer period than 10 days after the cessation of the injections. At the end of this period the weight of the uterus was only slightly decreased, while that of the vagina was slightly increased (groups XIII and XV) as compared with the weights of these organs in rats killed on the day after the last injection (groups XII and XIV). With the benzoate-butyrate, the maximum effect was still fully maintained on the 106th day after the last injection (group VIII).

With both hormones, the normal weight and size of the *uterus* was not obtained with any of the doses. Even with 300  $\mu$ g. (group IX) the large weight of the uterus was due to accumulation of secretion in the uterine lumen greatly distending the uterus, a similar condition to that observed during oestrus. Such a secretion greatly increases the weight of the uterus, while the control animals in group I were always killed during dioestrus, when this secretion is absent.

Furthermore, with all doses, metaplasia into squamous epithelium was observed in the uterine epithelium (the first stage of "oestrogenic precancerous" changes). This metaplasia, sometimes in a severe form, was still present in some rats even 106 days after the injections were ceased (group VIII). This means that not only physiological recovery but also the pathological effect was maintained and can be present as much as 15 weeks after the last injection.

One of the rats injected with 300  $\mu$ g. of the benzoate-butyrate developed pyometra in the greatly enlarged uterus (weight 4.3 g.), with adhesions to neighbouring organs. Such an effect has been found previously by other workers after injection of large doses of oestrogens.

In contrast to the uterus, the *vagina* returned to normal or in some groups (VIII and IX) was even larger and better developed than in normal control rats. In the rats killed on the next day or up to about two months after the last injection the vagina had a structure more or less typical of oestrus (cornified epithelial layer).

*Progestational changes in the vagina.* The most remarkable effect, however, occurred in the group of 4 rats killed 106 days (group VIII) after discontinuance of the injections: in two of these rats the appearance of the vagina was similar

to that found at metoestrus (with some cornification), but in the remaining two the epithelial layer was mucified—in one rat to a degree not far from that found during pregnancy.

From these results the following conclusions may be suggested, but confirmation on a larger number of ovariectomized animals is necessary:

(1) Oestrogenic compounds with a long period of action after discontinuing the injections (e.g. oestradiol benzoate-butyrate) may, towards the end of this period, produce a progestational instead of oestrous effect on the vagina.

(2) Since progesterone is naturally present in gonadectomized animals (e.g. in the adrenals [Beall & Reichstein, 1938; Beall, 1938]), the injected oestrogens, if present in the correct ratio with progesterone, might produce this progestational effect on the vagina.

(3) Probably, in the case of oestradiol benzoate-butyrate this ratio is reached toward the end of the after-period of "106 days rest".

(4) This explanation seems to us more probable than that oestrogens alone, without co-operation with progesterone, when injected in small amounts produce mucification, and when in larger amounts produce cornification of the vagina.

The latter theory, however, has been suggested by the workers who first noted the mucifying effect of very small amounts of oestrogenic concentrates or of purified oestrone [Robson, 1931; Robson & Wiesner, 1931-32; Meyer & Allen, 1932]. The latter authors also review the literature on the subject.

The *preputial glands* in most rats were slightly enlarged but did not return to normal.

#### *Effect on body weight, fat deposition and non-sexual organs*

In our previous experiments pure oestrone was injected in doses of 2-18  $\mu$ g. daily for 4 weeks [Korenchevsky *et al.* 1935] or 6  $\mu$ g. three times a week for 3 weeks [Korenchevsky & Dennison, 1936; Korenchevsky *et al.* 1936; 1937, 1 and 2] and the rats were killed on the day following the last injection. These doses of this compound did not produce constant changes in the non-sexual organs of ovariectomized females, with one exception—the thymus. The physiological involution of this organ, delayed in ovariectomized rats, was increased by oestrone. In some cases the weight of the liver was slightly decreased and that of the hypophysis slightly increased. A definite decrease in the gain in body weight and fat deposition was noted. Stunted growth after injections of oestrogens has been reported by several authors.

In the present experiments some of the effects observed were similar to those obtained previously, but other changes also occurred.

*Body weight and fat deposition.* As in our previous experiments, the di-esters produced stunted growth and decreased fat deposition in all groups—the larger the dose, the greater the effect. However, although during the injections the body weight remained stationary or increased less than in the controls, or even in some cases began to fall slightly, in the after-periods of "rest" the body weight and fat deposition began to return towards normal in spite of the persistence of the sex effect. With 18  $\mu$ g. these restorative changes were much more marked than with 90  $\mu$ g.

*Non-sexual organs.* The effect on the *thymus* (increased involution) was the same as in our previous experiments, but the enlargement of the *hypophysis* became more constant and much more pronounced. The larger the dose, the greater was the hypophyseal hyperplasia. With the benzoate-butyrate the hyperplasia increased during the first month after the end of the treatment

(groups IV and VII), but later restorative changes began to develop (groups V and VIII), while the sex effect was still fully maintained. The changes with the dipropionate (groups XIII and XV) were too small to suggest a definite decrease of hypophyseal hyperplasia 10 days after the end of the treatment.

A definite change in the actual weight of the *liver* was not always found during the period of injections, but in the after-periods of "rest" a considerable enlargement occurred (both in actual weight and that per unit of body weight) when small doses were given (groups IV, V and XIII). When the dose was increased up to 90  $\mu$ g. there was no significant change in the weight of the liver after the injections ceased (groups VII, XV). However, when the period of rest is longer, as the experiment with benzoate-butyrate shows (groups V and VIII), the enlargement of the liver decreases or disappears, in spite of the maintenance of the sex effect.

That the changes in the liver are significant is supported by the fact that, per unit of body weight, the weight of the liver was also increased in all the groups even during the periods of injections. It is clear, therefore, that during the period of the injections, or when large doses of the esters were injected the effect of oestrogens on the liver was veiled by the stunted growth of the animal, but the weights per unit of body weight and the enlargement of the organ after the end of treatment suggest a stimulating action of oestrogens on this organ, provided that histological investigation will confirm this conclusion.

The *kidneys* were enlarged in varying degree in all groups both in actual weight (except group IX) and those per unit of body weight.

In *adrenals and heart* no constant or pronounced changes were observed in the actual weights, but per unit of body weight there was an increase in most of the injected rats.

*Thyroids and spleen* showed no definite changes in weight.

#### *Comparison of the effects of female hormones on females with those of male hormones on males*

If the general results of previous experiments (of about 3 weeks' duration) performed by Korenchevsky and co-workers on castrated male rats injected with male hormones are compared with the present observations, some similarities and differences in the action of male and female hormones on males and females respectively can be noted. Thus, as judged by weights, the di-esters of oestradiol and the male hormones produced similar effects on sex organs, thymus, liver (with the reservations for oestrogens already mentioned) and kidneys. The two groups of hormones, however, in most cases influenced differently the body-weight, fat deposition, hypophysis and adrenals.

Thus the oestrogens do not act on females in the same way as the male hormones on males, although some similar properties are present in both groups.

#### SUMMARY

1. The effects of the dipropionate and benzoate-butyrate of oestradiol were studied on 99 ovariectomized rats.

2. With the doses used, the effects on the sex organs were maintained in the case of the dipropionate during the investigated period of 10 days without injections, while the action of the benzoate-butyrate had not begun to decline even on the 106th day after the last injection.

3. Complete restoration to normal weight and structure of the vagina but not of the uterus was obtained. Moreover, an important pathological change, squamous metaplasia, occurred in the uterine epithelium and was still present up to 106 days after cessation of the injections.

4. While the restorative effect on the sex organs in the case of oestradiol benzoate-butyrate was maintained at its maximum for more than 106 days, the effect on the structure of the vagina in this later stage of the period without injections may be changed from oestrous to progestational form (mucification of the vaginal epithelium).

5. Most probably this progestational effect on the vagina was due to co-operative action at this period of a small amount of oestrogenic hormone (liberated from the injected depot) with progesterone which has probably originated from the adrenals.

6. Body growth and fat deposition were depressed by the di-esters but a partial recovery took place in the period after the injections.

7. Involution of the thymus was hastened: hyperplasia of the hypophysis was present during and after the period of the injections: pronounced enlargement of the liver during some after-periods, and of the kidneys during and after the periods of injections was noted.

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# XLVII. THE MANIFOLD EFFECTS OF PROLONGED ADMINISTRATION OF SEX HORMONES TO FEMALE RATS

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IN his excellent book Simonnet [1937] has collected and analysed the extensive literature on the effects of oestrogenic hormones, while the papers on the effects on females of male hormones and their co-operative and antagonistic activities with oestrogens have recently been reviewed by Korenchevsky [1939].

Several workers have studied the effects of prolonged administration of oestrogenic hormones, but long duration experiments with male hormones on females are urgently needed.

In our previous papers [see references, Korenchevsky, 1937] we have studied the effects of male sex hormones on normal and spayed rats for the comparatively short period of 21 days. It seemed probable that certain weak and inconstant changes, produced especially in the non-sexual organs, might be more clearly defined if the period of administration of the hormones were prolonged. In the present experiments, therefore, the rats were injected for the comparatively long period of 3-3½ months, and, as will be shown in this paper, several aspects of the problem were made clear. Such an investigation is also of clinical importance, since the hormones are often applied for long periods and in large doses in order to obtain the desired results.

In the present experiments the following sex hormones have been investigated:

(1) The natural male hormones, i.e. those which are known to occur naturally in the female organism, namely androsterone [Callow & Callow, 1938] and *trans*dehydroandrosterone [Callow, 1936]. As the "female" activity of these hormones is weak [Korenchevsky *et al.* 1935; Korenchevsky & Dennison 1936, 1, 2], large doses of 7.5 mg. a week were given.

(2) Testosterone propionate was included as possessing the strongest female activity of all the known male hormones [Korenchevsky, 1937; Korenchevsky & Hall, 1937]. Medium (2.25 mg. a week) and large (7.5 mg. a week) doses were used.

(3) Of the oestrogens, oestradiol dipropionate was chosen because according to Miescher *et al.* [1938] it appears to be one of the most interesting esters of oestradiol, with a not too prolonged activity on oestrus and growth of the uterus, but of which otherwise little is known. Hormones with very prolonged activity (e.g. oestradiol benzoate-butyrate) have the disadvantage that, once they have been injected, their effects cannot be checked even if desired, and control over their physiological and pathological activities is lost for a very long period [Korenchevsky *et al.* 1939, 1].

Doses of 18, 90 and 200 µg. a week of oestradiol dipropionate were used alone or simultaneously with the male hormones, in order to investigate their combined effects.

(4) In one group of our experiments, progesterone was given simultaneously with testosterone propionate and large doses of oestradiol dipropionate, the main object of this being to study the property of progesterone of preventing the pathological changes produced by oestrogens in the sexual [Korenchevsky & Hall, 1938, 1] and perhaps in some other organs.

Details of the results of histological investigation will be published elsewhere, a few statements only being included in this paper. Some preliminary results have already been published [Korenchevsky & Hall, 1938, 2].

### Technique

The general arrangement of the experiments is clear from Tables I and II. A total of 88 rats was used: 9 normal and 13 spayed animals were kept as controls, and 4 normal and 62 spayed animals were injected with the hormones. The rats in Table I were injected for an average of 87 days, their average age when killed being 131 days. In Table II the average age of the spayed rats in groups XI–XVI was 150 days and that of the normal rats in groups X, XVII and XVIII was 201 days, all the rats having been injected for an average of 109 days. Ovariectomy was performed at the age of 21–24 days.

The male hormones and oestradiol dipropionate were supplied by Messrs Ciba Ltd., progesterone by Messrs Organon Ltd. The hormones were dissolved in sesame oil and were injected: androsterone, *transdehydroandrosterone* and testosterone propionate five times a week, oestradiol dipropionate three times a week (except the dose of 200  $\mu$ g. given once a week), progesterone six times a week. The weekly doses are given in the tables. Pure sesame oil was injected into the control rats.

For economy of space the data per unit of body weight have not been tabulated, but will where necessary be referred to in the text.

The other details of experimental technique were the same as in our previous papers.

Table I. *Effects of androsterone and transdehydroandrosterone injected alone or simultaneously with oestradiol dipropionate on actual weights of organs of spayed female rats*

	Spayed rats injected weekly with											
	Controls injected with oil		Oestradiol dipropionate		Androsterone, 7.5 mg.			Dehydroandro- sterone, 7.5 mg.				
					Alone	+ Oest- radiol dipro- pionate	+ Oest- radiol dipro- pionate	Alone	+ Oest- radiol dipro- pionate			
	Normal rats	Spayed rats	18 $\mu$ g.			18 $\mu$ g.	90 $\mu$ g.		18 $\mu$ g.	90 $\mu$ g.		
			I	II			III			IV	V	VI
Uterus (mg.)	448	34	266	283	59	327	357	63	366			
Vagina with clitoris (mg.)	292	190	332	350	312	416	440	253	366			
Preputial glands (mg.)	92	62	74	54	205	126	104	132	84			
Adrenals (mg.)	78	70	85	62	57	73	84	57	63			
Hypophysis (mg.)	15.4	12.3	37.3	98.0	10.8	30.3	48.0	11.8	55.5			
Thymus (mg.)	261	380	253	178	274	223	125	344	172			
Liver (g.)	8.66	8.93	8.17	7.61	11.39	10.01	8.96	9.81	8.90			
Kidneys (g.)	1.71	1.61	1.78	1.56	2.25	2.11	2.01	1.64	1.70			
Spleen (mg.)	515	618	465	446	739	481	630	824	376			
Heart (mg.)	730	769	685	623	928	759	723	768	644			
Abdominal fat (g.)	18	20	11	9	20	13	9	19	12			
Final body wt. (g.)	230	308	218	205	339	257	228	308	225			
Gain in body weight (g.)	126	169	82	73	199	103	112	165	76			
No. of rats in group	3	8	3	5	4	4	3	4	4			

Table II. *Effects of testosterone propionate injected alone or simultaneously with oestradiol dipropionate and progesterone on actual weights of organs of female rats*

Organs	Spayed rats injected weekly with								
	Controls injected with oil		Oest-radiol dipropionate 0.2 mg.	Testosterone propionate 2.25 mg.	Testosterone propionate 7.5 mg.	+ oest-radiol dipropionate 0.2 mg.	Testosterone propionate 2.25 mg. + progesterone 4.5 mg. + oest-radiol dipropionate 0.2 mg.	Normal rats injected weekly with testosterone propionate	
	Normal rats	Spayed rats						XVII 0.75 mg.	XVIII 7.5 mg.
X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	
Uterus (mg.)	526	33	326	262	395	511	337	383	3295
Vagina with clitoris (mg.)	324	175	311	471	534	484	463	522	611
Preputial glands (mg.)	85	49	50	198	196	203	121	141	245
Adrenals (mg.)	66	63	53	50	62	64	60	47	66
Hypophysis (mg.)	17.6	11.5	132.3	10.6	10.9	60.2	47.2	11.9	11.6
Thymus (mg.)	155	327	128	123	26	75	55	129	59
Liver (g.)	8.78	8.69	6.77	10.42	11.18	7.91	7.65	9.58	9.10
Kidneys (g.)	1.74	1.54	1.52	2.08	2.54	1.86	1.88	1.84	2.51
Spleen (mg.)	642	503	340	913	820	429	387	841	716
Heart (mg.)	816	704	596	935	899	621	632	963	1065
Abdominal fat (g.)	19	22	6	18	12	8	8	17	9
Final body wt. (g.)	273	323	192	314	290	203	221	303	273
Gain in body wt. (g.)	67	161	13	139	73	64	56	92	68
No. of rats in group	6	5	9	7	4	6	9	2	2

*Ovariectomized rats*

*Effect of ovariectomy.* In this paper the changes observed after ovariectomy will be only briefly summarized, the conclusions being drawn from the results of our previous and present experiments on rats. The only really striking changes, following ovariectomy performed before sexual maturity, occur in the sex organs, which become atrophic. Of the non-sexual organs, a definite change occurs in the thymus, the physiological involution of which becomes delayed, i.e. the gland is larger than in control rats of the same age. The gain in body weight increases after ovariectomy, this increase being only partly due to the slight increase in the amount of body fat. Unlike castration, ovariectomy did not produce any pronounced or constant changes in the actual weights of adrenals, hypophysis, liver, kidneys, spleen and heart. The weights of these organs when calculated per unit of body weight, however, were in most cases slightly decreased, which is to be explained by the greater body weight of the spayed rats as compared with that of the normal intact animals.

In males, on the other hand, the gain in body weight decreases after castration. Therefore the effects of gonadectomy on the weights of the organs calculated per unit of body weight are more similar in both sexes with liver, kidneys, spleen and heart than they appear from the actual weights, but with hypophysis and adrenals the differences are more pronounced.

*Effect of the hormones on sex organs*

*Oestradiol dipropionate* (Table I, groups III and IV; Table II, group XII). With all doses the weight of the vagina reached normal or even supernormal level, but complete restoration of the uterus was not obtained. The largest

uterus was produced by the largest dose of 200  $\mu$ g. (group XII). No constant changes were found in the preputial glands.

In most cases the vagina showed oestrous cornification of the greatly enlarged epithelial layer, or in some cases swollen epithelial cells with dropsical vacuolation. The uterus was of abnormal fibrotic structure. With the smaller doses (18 and 90  $\mu$ g.) the epithelial cells were high columnar and in only one rat out of 8 contained a few very small patches of cells with squamous metaplasia (first stage of "precancerous" changes produced by oestrogens). With 200  $\mu$ g. the metaplasia of the uterine epithelium was present in about 44% of rats, in some cases being very pronounced and involving the whole layer, with cornification of the upper row of cells.

*Androsterone and dehydroandrosterone* (Table I, groups V and VIII). Both these hormones had only a very weak effect on the uterus, not significantly greater than that produced in our previous experiments of only 21–27 days' duration [Korenchevsky *et al.* 1935; Korenchevsky & Dennison, 1936, 1]. A much greater effect, especially with androsterone, was obtained on the vagina and preputial glands. Dehydroandrosterone, as in our previous experiments [Korenchevsky & Hall, 1937], caused mucification of the vaginal epithelium.

The addition of oestradiol dipropionate had a definite co-operative effect (as judged by weights of the organs) on the activity of androsterone, producing even superdevelopment of the vagina (Table I, groups VI and VII), while the co-operative effect with dehydroandrosterone was much less definite, especially on the vagina (group IX). After these simultaneous injections the effects of the oestradiol predominated on the structure of both vagina and uterus: the vagina in most cases showed oestrous changes, while fibrotic and other changes typical for oestradiol were seen in the uterus.

It is very remarkable that the *metaplastic changes in the uterine epithelium* occurred more frequently and were generally of more pronounced degree when androsterone, and apparently also dehydroandrosterone, were injected simultaneously with oestradiol dipropionate, than when the latter hormone was injected alone. With simultaneous injections the metaplastic changes were present respectively in 83% (groups VI and VII) and 50% (group IX), as compared with 13% in groups III and IV (oestradiol alone).

However, before making a definite statement that androsterone and *trans*-dehydroandrosterone increase the "oestrogenic precancerous" changes, it will be necessary to repeat the experiment on a greater number of animals.

*Testosterone propionate*. When this hormone was injected alone (Table II, groups XIII and XIV), a large uterus and supernormal development of the vagina and preputial glands were obtained.

The structure of both the vagina and uterus was similar to that previously described in experiments of 21 days' duration [Korenchevsky & Hall, 1937], the chief features being: in the vagina—mucification of the epithelium: in the uterus—an increased amount of fibrous tissue in the stroma, hypertrophied muscle cells, and (with the large dose) progestation-like lace foldings of the mucosa, which were, however, much more pronounced and more closely reminiscent of those found during pregnancy than those obtained in our previous experiments of short duration.

The addition of *oestradiol dipropionate* (group XV) was followed by better growth of the uterus, but severe squamous metaplasia of the uterine epithelium with cornification was present in half of the rats. Testosterone propionate in the dose used (2.25 mg. a week) was unable to prevent these pathological effects of oestrogens. The effect of oestradiol also overruled that of the male hormone in



the vagina, in which the changes were of the oestrous type, though in some cases irregular.

When, however, in another experiment (which will be described in detail elsewhere) a large dose of 7.5 mg. a week of testosterone propionate and a smaller dose (90  $\mu$ g. a week) of oestradiol benzoate-butyrate were injected, the development of the metaplastic changes was completely prevented, while in the uterus and vagina the effect of testosterone propionate (abortive progestational changes) was present in most cases.

*The addition of progesterone* to the combined injections of testosterone propionate and oestradiol dipropionate (group XVI) had a definite preventive action on these pathological changes: metaplasia was present only in 1 rat out of 9, and was of a very weak degree. We have shown previously [Korenchevsky & Hall, 1938, 1] that progesterone was able to prevent these metaplastic changes in the uterine epithelium produced by pure oestradiol, and have given the literature relating to the subject.

The special pictures of the uterus with abortive epithelial foldings and of the vagina with vacuolated, swollen epithelium in rats of group XVI will be described elsewhere.

It is also noteworthy that the addition of progesterone was followed by the development of a smaller uterus (group XVI) as compared with that obtained in group XV.

#### *Significance of varying amount of body fat in calculations per unit of body weight*

The amount of body fat in the experimental animals varied considerably under the influence of the injections. Therefore there is a possibility that misleading conclusions might be drawn, if the weights of the non-sexual organs calculated per unit of body weight of fat rats (e.g. control groups, especially spayed rats) are compared with those of thin animals (e.g. those injected with oestradiol). The depots of inert adipose tissue require less activity from the organs regulating metabolism than do active tissues such as glandular, muscular etc. Consequently different requirements might influence differently the size and weight of the "metabolic" organs.

When studying the non-sexual organs, therefore, we have calculated the weights not only per unit of final body weight, but also per unit of "fat-free" body weight. This latter weight was obtained approximately by subtracting the total body fat from the final body weight (given in the tables) assuming that the abdominal fat (also given in the tables) represents about 30–32% of the total fat in the body (for details see our previous paper [Korenchevsky *et al.* 1939, 2]). These calculations, however, gave results differing only in degree from the usual calculations per unit of body weight, without changing the essence of the conclusions.

#### *Effect on gain in body weight and fat deposition*

Growth, as judged by gain in body weight, was slightly stimulated by androsterone (group V), little or not at all affected by dehydroandrosterone and small doses of testosterone propionate (groups VIII and XIII) and depressed by oestradiol dipropionate (considerably, groups III, IV and, especially, XII) and large doses of testosterone propionate (slightly, group XIV).

The amount of body fat was considerably decreased by oestradiol dipropionate (the larger the dose the greater the decrease) and to a less degree by large doses of testosterone propionate, but unchanged by the other hormones or doses.

The remarkable neutralizing effect of the male hormones on oestrogens was again demonstrated by their power, when simultaneously injected, of preventing in most cases, and sometimes to a considerable extent, the depressing effect of the female hormone on body weight and fat deposition (groups VI, VII, IX, XV, XVI).

*Effect of the hormones on non-sexual organs*

**Adrenals.** The actual weights of these glands were slightly changed by oestradiol dipropionate, the smallest dose causing an increase (group III), the other doses a decrease (groups IV and XII). When calculated per unit of body weight, the weights showed with all doses a constant increase of 30–70%. This suggests that in spite of the evidence of actual weights, oestradiol dipropionate may have in reality some stimulating action on the adrenals, but fibrous degenerative changes found in the cortex did not support this suggestion.

The male hormones when injected alone (groups V, VIII and XIII, but not the large dose of testosterone propionate, group XIV) in most cases decreased both the actual weights of the adrenals and those per unit of body weight. Thus, in these prolonged experiments the effects of these hormones were similar in females and in males. In experiments of shorter duration on females [Korenchevsky *et al.* 1935; 1936; 1937, 1; Korenchevsky & Dennison, 1936, 1] the effects were similar but with some doses less definite and constant. Testosterone dipropionate also produced smaller adrenals [Korenchevsky *et al.* 1939, 3].

When male and female hormones were injected simultaneously (groups VII, IX, XV and XVI) the activity of the female hormone in most cases changed the effect of the male compounds, the adrenals becoming larger than those of rats injected with the respective male hormones alone. A similar effect was noted with some hormones in our previous experiments (mentioned above) of shorter duration.

**Hypophysis.** As with other oestrogens, oestradiol dipropionate produced hyperplasia of the hypophysis, the larger the dose the greater being the degree of hyperplasia (groups III, IV and XII). It is remarkable that while male hormones have no definite effect upon the weight and size of the gland (groups V, VIII, XIII and XIV), they can to some extent prevent the hyperplastic effect of oestrogens (groups VI, VII, IX, XV). If in addition progesterone is given (group XVI), the neutralizing effect appears to be even greater; the hypophyses of two rats in this group weighed only 19.8 and 25.6 mg. as compared with an average of 132 mg. with oestradiol alone (group XII). The weight of the hypophysis was thus restored nearly to normal, the average weight in normal controls being 17.6 mg.

**Liver, kidneys, heart and spleen.** With a few exceptions, the character of the changes in these organs was similar.

**Oestradiol dipropionate** (groups III, IV and XII) caused a decrease (considerable with large doses) in the actual weights of these organs, except the kidneys, while the weights of all of them per unit of body weight were increased. Therefore, as in the case of the adrenals, the ratio of the weights of these organs to body weight was greater than in control ovariectomized rats and often similar to that in control normal animals.

On this basis the influence of oestradiol on these organs cannot be considered as a true depressing effect, but may even suggest some stimulation, hidden by the effect of the stunted growth of the rats on the weight and size of these organs provided that histological investigation confirms this conclusion. This is also supported by experiments of shorter duration (21 days) with oestradiol dipropionate and oestradiol benzoate-butyrate [Korenchevsky *et al.* 1939, 1], when

the weights, both actual and per unit of body weight, of liver and kidneys were increased by those doses which did not cause much depression of body weight.

On the other hand, the effect of *male hormones* on liver, kidneys, heart and spleen appears to be definitely stimulating, both the actual weights and those per unit of body weight being increased (groups V, VIII, XIII, XIV). Even in experiments of shorter duration, similar results were obtained in some cases [e.g. Korenchevsky *et al.* 1939, 3].

This stimulating effect is further demonstrated by the property of androsterone and testosterone propionate of at least partly preventing the depressing effect of oestradiol on the actual weights of these organs (groups VI, VII, XV). The addition of progesterone (group XVI) does not increase this effect of testosterone propionate.

The weakest stimulant in this respect amongst the male hormones was dehydroandrosterone, which had a definite effect on liver and spleen, but not on heart and kidneys. It also possessed in only a very weak degree the property of preventing the changes produced by oestradiol.

*Thyroids.* The weights of these organs were unchanged.

*Comparison of the effects of the sex hormones on the non-sexual organs in females with those in males*

As shown above, gonadectomy does not produce such constant and definite effects on the majority of the non-sexual organs in females as in males. In spite of this, however, the effects of the male and female hormones on adrenals, hypophysis, liver, kidneys, spleen, heart, fat deposition and gain in body weight were similar in both sexes (compare the present results with those obtained in similarly arranged experiments on males [Korenchevsky *et al.* 1939, 2]). The remarkable property of male hormones of more or less neutralizing the effects of the female hormone was also present in both sexes. It should be noted, however, that while in males the weights of liver, kidneys, heart and spleen were restored to normal by male hormones, in the female these hormones produced super-normal development of these organs.

*Effect of testosterone propionate on normal rats*

Only six rats were used in this preliminary experiment, two in each of the injected groups and two control animals. The results, however, were constant, clear-cut and in many respects in good agreement with those of our previous experiments of shorter duration (21 days) [Korenchevsky *et al.* 1937, 2].

In normal females (groups XVII and XVIII) the effect of testosterone propionate on most of the organs investigated was similar in character to that in ovariectomized rats, but usually differed in degree. The following changes in weight (both actual and per unit of body weight) were found: a "gigantic" uterus with the large dose, and greatly enlarged vagina and preputial glands with both doses; decrease in weight of the adrenals with the small dose and no change with the large dose; decreased weight of hypophysis, and enlargement, varying in degree, of liver, kidneys, spleen and heart. The small dose caused increased gain in body weight but no definite change in fat deposition, while with the large dose fat deposition was decreased but the body weight remained unchanged. As in ovariectomized rats, therefore, the large dose affected fat deposition and body weight more than the small dose.

Some unusual features of the changes in the uterus and vagina must be mentioned. With the small dose there was a decrease in size of the uterus (group XVII), but the structure was normal, with few and low foldings of the

mucosa. The small size of the uterus might be explained by the depressing effect of testosterone propionate on the secretion of gonadotropic hormone from the hypophysis, this dose of the hormone being unable to counterbalance the depressing effect by direct stimulation of the uterus. Analogous results were obtained previously [Korenchevsky & Hall, 1939] in normal male rats after injection of androsterone and *transdehydroandrosterone*, and the mechanism of this phenomenon was discussed in detail (p. 376). The increase in size of the vagina, however, does not accord with this theory, and therefore at present it is difficult to find a satisfactory explanation of the decrease in size of the uterus, especially on the basis of the results of an experiment on two rats only.

The "gigantic" uterus (group XVIII), produced by the large dose, was distended with secretion, the distension being reminiscent of that during oestrus (the columnar epithelium was normal and the myometrium hypertrophied). The epithelial layer of the vagina was narrow with narrow, mucified cells.

#### SUMMARY

1. Androsterone, *transdehydroandrosterone*, testosterone propionate and oestradiol dipropionate, alone, or each of the male hormones in combination with oestradiol dipropionate (in one group also with progesterone) were injected into ovariectomized and normal rats for a period of about 3½ months.

#### *Effects on sex organs of ovariectomized rats*

2. With oestradiol dipropionate alone, the vagina was restored to normal or supernormal weight, with normal (usually "oestrus") structure, but not even a weekly dose of 0.2 mg. was able to produce normal size and weight of the uterus, although this dose caused pronounced pathological changes in the structure of this organ.

3. The weak effects of androsterone and *transdehydroandrosterone* on the uterus were not significantly increased, as compared with experiments of shorter duration, but the vagina and especially the preputial glands were considerably hypertrophied.

4. There was a co-operative effect between oestradiol dipropionate and androsterone or (to a lesser degree) *transdehydroandrosterone* on the weight and size of the sex organs, but at the same time the number of rats affected with squamous metaplasia of the uterine epithelium (first step of "precancerous" oestrogenic effect) was increased and this condition became more severe.

5. Testosterone propionate produced pronounced progestational changes in the uterus and less strong ones in the vagina.

6. Testosterone propionate had a co-operative effect with oestradiol dipropionate on the weight and size of the uterus, but the occurrence and degree of epithelial squamous metaplasia in this organ were about the same as with the female hormone alone.

7. If, however, progesterone was added to the combination of male and female hormones, or a large dose of testosterone propionate was injected with oestradiol, these "precancerous" changes in the uterine epithelium were prevented to a remarkable degree.

#### *Effects on non-sexual organs*

8. Male hormones caused decrease in the weight and size of the adrenals of females similar to that previously described in male rats, while the effect of the female hormone was characterised by peculiar pathological changes. Enlarge-

ment of the adrenals in most cases followed simultaneous injections of male and female hormones.

9. The tumour-like hyperplasia of the hypophysis produced by the oestrogens was neutralized to a considerable extent (in two rats almost to normal) by male hormones.

10. Male hormones caused hypertrophy (in most cases exceeding normal level) of liver, kidneys, spleen and heart, but the female hormone produced either a slight decrease in their actual weights, or (kidneys) no change.

11. Oestradiol dipropionate produced stunted growth and decreased fat deposition, but with the male hormones this depressing effect was absent or (with the large dose of testosterone propionate) slight.

12. When injected simultaneously the male hormone more or less completely neutralized the effect of the oestrogen on liver, kidneys, heart, spleen, fat deposition and body weight.

#### *Effect on normal rats*

13. The effects produced by testosterone propionate on the weight and size of most organs of normal rats were in general similar to those in ovariectomized rats, in most cases differing in degree. A remarkable feature was the "gigantic" uterus distended with oestrus-like secretion.

14. Prolonged administration of male sex hormones produced in general similar results in both sexes.

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# XLVIII. PRELIMINARY EXTRACTION OF GONADOTROPHIC PRINCIPLE FROM PREGNANT MARE SERUM

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THE serum of the mare contains a relatively high concentration of gonadotrophic material during the second and third months of pregnancy [Cole & Hart, 1930]. This substance resembles anterior pituitary extracts in causing development of the follicles, luteinization and interstitial cell proliferation in the ovary of normal and hypophysectomized rats [Evans *et al.* 1933, 1, 2; Rowlands, 1938] while in the hypophysectomized male spermatogenesis is maintained or restored and the interstitial cells are stimulated [Evans *et al.* 1933, 3; Liu & Noble, 1939]. As a result of gonad stimulation secondary effects are produced in both sexes.

In the present paper a simple method is described for separating the active principle from the bulk of inert material in the serum

## *Methods*

Serum was obtained from the freshly clotted blood of mares at a suitable stage of pregnancy. It was stored at 0° and maintained at as low a temperature as practicable during all procedures.

The gonadotrophic activity was assayed by the increase in weight produced in the ovary of immature (40–50 g.) female rats. Since the supply of serum was limited groups of only 3 rats could be used for each test. Daily injections were made for 5 days and the animals killed on the sixth. The organs were dissected, fixed in Bouin's fluid and weighed from 70% alcohol.

## *Results*

*Precipitation with salicylsulphonic acid.* An attempt was made to discover a method of removing the inactive material in the serum from the active substance. According to a British patent (specification No. 440,530 (19. ii. 1935)) protein precipitants such as picric acid, salicylsulphonic acid and trichloroacetic acid have been used for this purpose. Preliminary experiments in which picric acid or salicylsulphonic acid was added to the serum to complete precipitation gave inactive filtrates, but by suitably adjusting the concentration, and consequently the pH of the salicylsulphonic acid, it was found possible to obtain the required separation. The following standard method was employed for each precipitation, the concentration of acid being altered systematically. To 20 ml. of serum at 0° varying amounts between 1 and 2 ml. of 33.3% salicylsulphonic acid were added with constant stirring. The mixture was kept in the refrigerator for about 10 min., and was then centrifuged in cooled buckets. The clear supernatant fluid was decanted: its pH was determined by glass electrode and it was neutralized immediately with 2N NaOH. The precipitate was washed with 5 ml. of acid solution. After centrifuging the supernatants were pooled and adjusted to pH 7.5 using bromothymol blue as indicator.

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<sup>2</sup> Working on a grant from the Medical Research Council.

The results are summarized in Fig. 1, the weights of the ovaries being plotted against the *pH* of the different precipitations. Each test was performed on the equivalent of 2 ml. of the original serum which gave ovaries of 316 mg. when tested. From an examination of the curve it can be seen that the yield of activity

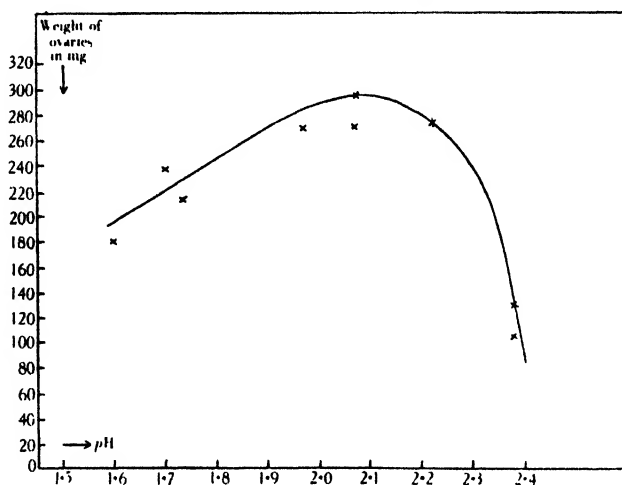


Fig. 1. Relationship between yield of gonadotrophic substance in salicylsulphonic acid supernatant and *pH*.

at *pH* 1.9–2.2 is almost 100%. At *pH* > 2.2 the decrease in potency is very rapid. Below *pH* 2.2 it is more gradual and probably due to destruction by the acid rather than to partial precipitation of the active material. It is important to use fresh yellow serum for this method since haemolysed serum requires much more salicylsulphonic acid to obtain the required *pH* and even then there is a considerable loss of potency in the extraction. A similar series of precipitations was carried out with normal horse serum to obtain the quantitative relationship between the *pH* of the precipitation and the weight of total solids in the supernatant. The supernatant solutions after dialysis to remove salicylsulphonic acid and salts were therefore evaporated to dryness and the solid content determined.

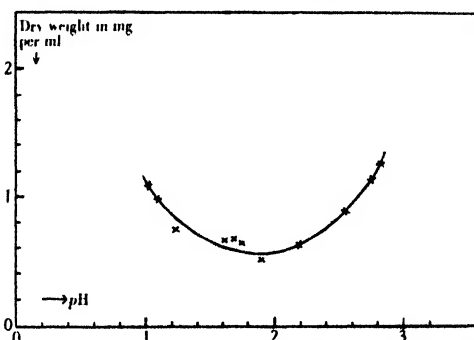


Fig. 2. Relationship between yield of dry substance in salicylsulphonic acid supernatant and *pH*.

From Fig. 2 it may be seen that a minimum yield of solid material is obtained from the supernatant at that *pH* where there is a maximum yield of gonadotrophic hormone, when pregnant mare serum is used.

Further concentration of the gonadotrophic principle was obtained following dialysis of the supernatant in a collodion thimble for 15–20 hr. at 4°. No loss of activity followed this treatment, and the solution after dialysis was evaporated to dryness *in vacuo* at low temperature, or precipitated with 80 % acetone. The yield of dry substance after such procedure was 100–150 mg. per 100 ml. of serum. It was found that if the solution after dialysis was concentrated to a small volume a precipitate was formed which could be removed by centrifuging. As this precipitate contained no activity the weight of dry active material could be reduced to 30–50 mg. per 100 c.c. of serum.

*Acetone precipitation.* A small amount of the active material was obtained by the above process and treated by fractional precipitation with acetone. The active substance was quite soluble in 50 % acetone or alcohol, but was precipitated quantitatively in concentrations above 80 % acetone. In one experiment a solution containing 6.5 mg. of the dry powder per ml. was adjusted to pH 7.5. Following the addition of an equal volume of acetone the precipitate was allowed to flocculate in the cold and was then centrifuged. The precipitate (PMS 8083) on assay was found to contain from 20 to 30 % of the total activity. The acetone concentration of the supernatant fluid was raised to 80 % and the further precipitate washed with acetone and ether and dried *in vacuo*. This powder (PMS 8082) contained 70 to 80 % of the total gonadotrophic activity. This experiment was repeated using a more dilute solution (PMS 83—3 mg. per ml.) since the activity in the 50 % acetone precipitate might be due to adsorption. An equal volume of acetone added to this solution only produced a faint opalescence and no definite precipitate was obtained until the pH was brought to 5.0. There was no activity in this precipitate but that obtained with 80 % acetone contained 100 % (PMS 831).

The dry weight of the final products represented 0.13 mg. and 0.09 mg. per ml. of the original serum respectively. Table I gives the activities of these various

Table I

No.	Preparation	Dose per rat			
		Equiv. to ml. of serum ml.	Wt. mg.	Ovaries mg.	Uterus mg.
PMS 801	Salicylsulphonic acid supernatant	1	(1)	64	154
PMS 8081	Insoluble part of supernatant	5	—	15	30
PMS 8083	50 % acetone precipitate	1	—	26	147
PMS 8082	50 % acetone supernatant	1	0.13	44	107
PMS 83	Salicylsulphonic acid supernatant	1	(0.25)	66	267
PMS 831	50 % acetone supernatant	1	0.09	67	141

samples, the comparatively low ovary weights being due to the use of haemolysed serum and consequent loss of activity during the salicylsulphonic acid precipitation. The final product is a stable powder readily soluble in water. In concentrations of 3 mg. per ml. it gives neither Millon nor biuret tests. Since these tests are faintly positive when applied to serum proteins in the same concentration, this indicates that the final material is partly non-protein.

Lack of material prevented further research into this acetone fractionation but it appears that the optimal conditions for precipitation are dependent on the pH and on the concentration of material.

Under the conditions described it is possible, therefore, to accomplish an approximately 1000-fold concentration (on the basis of total solids) when compared with the crude serum, by a relatively simple and rapid chemical process.



In spite of the marked purification which can be produced, the greater part of the product is inactive material since the above process yields approximately the same weight of dry substance when applied to non-pregnant horse serum.

#### DISCUSSION

A number of chemical procedures have been described for the purification of the gonadotrophic factor of pregnant mare serum. Goss & Cole [1931] fractionated pregnant mare serum by precipitation with sodium sulphate but only obtained a 10-fold purification. Evans *et al.* [1933, 4] used acetone-ammonia extraction of a solution of the acetone precipitate from the serum. This was applied by Catchpole & Lyons [1934] to fresh serum without any appreciable separation from the serum proteins. Later Evans *et al.* [1933, 5] obtained a purer product by adsorption of the same material on aluminium hydroxide; this procedure was modified and adapted to fresh serum by Gustus *et al.* [1936]. Finally, Evans and his co-workers [1936] claimed to effect a separation of the follicle-stimulating and luteinizing fractions by fractional precipitation with ammonium sulphate. A laborious method of fractional precipitation with acetone or alcohol designed by Cartland & Nelson [1937] yielded a 130- to 1800-fold purification of the active principle. Severinghaus *et al.* [1938] obtained promising results using the ultracentrifuge. The results here described indicate that salicylsulphonic acid precipitation of the inert material is a simple and rapid means of concentration. The further purification following dialysis, and the fractional precipitation with acetone yields the active substance in a form in which it would appear to be well suited for further chemical study.

#### SUMMARY

A method is described by which a purified fraction of gonadotrophic hormone may be obtained from pregnant mare serum, the essential steps being the precipitation of the inert serum proteins by a suitable concentration of salicylsulphonic acid, and subsequent purification by dialysis and fractional precipitation with acetone. The material obtained was a white powder readily soluble in water, and represented approximately a 1000-fold purification of the hormone in terms of solid content of original serum.

We wish to record our gratitude to Dr A. S. Parkes of the Medical Research Council Staff and Mr F. Day of the Cambridge School of Agriculture for supplies of pregnant mare serum; also to Prof. E. C. Dodds for his interest and criticism.

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# XLIX. SYNTHESIS OF STEROID GLUCURONIDES

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*(Received 27 January 1939)*

THE steroid glucuronides have acquired fresh interest since Marrian and his co-workers [Cohen & Marrian, 1936; Cohen *et al.* 1936] succeeded in isolating oestriol glucuronide from pregnancy urine, and Venning & Browne [1936] were able in a similar way to recover pregnandiol glucuronide from the urine of pregnant women. According to Marrian's data oestriol glucuronide is about 28 times weaker as an oestrogen than free oestriol. There is evidence that pregnandiol glucuronide is the form in which the corpus luteum hormone, progesterone, is eliminated; the latter cannot be condensed with glucuronic acid unless it is previously reduced to the hydroxylated pregnandiol.

The "male" sex hormones are also eliminated in urine in combined forms, although it is not yet known whether the excreted compounds are also glucuronides.

Glucuronides of steroids have not yet been made synthetically; in the present paper the synthesis of the following is recorded: methyl dehydroandrosterone-triacetylglucuronate; dehydroandrosterone-glucuronic acid; methyl testosterone-triacetylglucuronate; methyl  $\alpha$ -oestradiol-3-benzoate-17-triacetylglucuronate;  $\alpha$ -oestradiol-17-glucuronic acid; methyl oestrone-triacetylglucuronate; methyl cholesterol-triacetylglucuronate.<sup>1</sup>

The synthetic preparation of glucuronides has become possible, since Goebel & Babers [1935] succeeded in preparing methyl acetobromoglucuronate, which is the necessary starting-point for the synthesis of these substances. When methyl acetobromoglucuronate and the appropriate sterol derivative are shaken in an indifferent medium, such as benzene with silver carbonate, glucuronide formation readily occurs.

The methyl steroid-triacetylglucuronates crystallize well. They are soluble in organic solvents, such as benzene and chloroform, and fairly readily soluble in hot absolute alcohol, from which they may be recrystallized.

The barium salts of the steroid-glucuronic acids can be prepared by hydrolysis of the acetylated methyl esters with baryta solution, and afford the steroid-glucuronic acids on treatment with acids. Dehydroandrosterone-glucuronic acid and  $\alpha$ -oestradiol-17-glucuronic acid prepared in this way are characterized by their solubility in dilute alcohol; they are somewhat soluble also in boiling water.

Dehydroandrosterone-glucuronic acid can be recrystallized from absolute alcohol.  $\alpha$ -Oestradiol-glucuronic acid crystallizes from very dilute alcohol with 1.5 mol. of water of crystallization, which are removed only on drying in a high vacuum at 115–120°. Also even after repeated recrystallization  $\alpha$ -oestradiol-17-glucuronic acid always contains 0.86–1.1% of ash; further purification of the substance is accompanied by heavy losses of material.

<sup>1</sup> The hormones necessary for this investigation were very kindly placed at my disposal by th. N. V. Organon, Oss (Holland). According to a private communication, Dr Luchs, of the Research Department N. V. Organon, Oss, tried to make esters and ethers of oestrone in 1934. This research could not be continued on account of Dr Luchs' illness.

## EXPERIMENTAL

I. *Methyl dehydroandrosterone-triacetylglucuronate*. Methyl acetobromoglucuronate (700 mg.) and dehydroandrosterone (500 mg.) were dissolved in benzene (25 ml.) and the solution shaken for 24 hr. with 1 g. of dry  $\text{Ag}_2\text{CO}_3$ . The solution, which was free from bromine, was centrifuged and the residue extracted repeatedly with warm benzene. The combined clear liquors from the centrifuge were evaporated *in vacuo* to a syrup, which crystallized spontaneously on rubbing with a little absolute alcohol. After standing in the ice chest for several hours, the crystals were filtered off, washed with a little cold alcohol and dried. Yield: 255 mg. (24 %), M.P.  $193\text{--}196^\circ$ ,<sup>1</sup> unchanged after recrystallization from absolute alcohol. (Found: C, 63.88; H, 7.13 %;  $\text{C}_{32}\text{H}_{44}\text{O}_{11}$  requires C, 63.54; H, 7.33 %.) The compound had  $[\alpha]_D^{20} -19.7^\circ$  ( $c=0.0033$  in  $\text{CHCl}_3$ ) or  $-16.2^\circ$  ( $c=0.0063$  in benzene).<sup>2</sup>

220 mg. were dissolved in 20 ml. of hot absolute methyl alcohol and to the hot solution were added 4 ml. of 0.4 N  $\text{Ba}(\text{OH})_2$ . A fine, white precipitate was soon formed. The mixture was boiled for 45 min. under a reflux (protected by a soda-lime tube), cooled, treated with 20 ml. of water and kept at  $0^\circ$  for several hours. The crystalline precipitate, consisting of fine needles, was filtered off, washed well with water and dried. Yield: 178 mg. (92 %).

II. *Dehydroandrosterone-glucuronic acid*. 172 mg. of the above Ba salt were digested on the water bath for 30 min. with 1.3 ml. of 0.25 N  $\text{H}_2\text{SO}_4$  and 5 ml. of 50 % methyl alcohol when the coarsely powdered Ba salt of the condensed glucuronic acid was replaced by finely divided  $\text{BaSO}_4$ . The warm suspension was centrifuged and the residue repeatedly extracted with warm 50 % methyl alcohol. The combined centrifugates were evaporated in a vacuum to incipient turbidity. On cooling, the solution soon began to deposit a very finely divided crystalline precipitate, which was filtered off after 24 hr., washed with ice-cold water containing methyl alcohol and dried in a vacuum over phosphorus pentoxide. It formed irregular scales with a mother of pearl lustre. Yield: 114 mg. (76 %).

After recrystallization from absolute alcohol, thick, glittering fragments of prisms were obtained with M.P.  $253\text{--}254^\circ$ . Two further recrystallizations from absolute alcohol afforded 49 mg. melting at  $254\text{--}257^\circ$ . Finally the substance was recrystallized from 3.5 ml. of 80 % alcohol, when the M.P. rose to  $262\text{--}264^\circ$  (constant). The crystals became yellow at about  $185\text{--}190^\circ$ , opaque at about  $210^\circ$  and finally melted with decomposition. Yield of pure substance: 25 mg. (Found: C, 64.05; H, 7.79 %.  $\text{C}_{25}\text{H}_{36}\text{O}_8$  requires C, 64.62; H, 7.82 %.)

III. *Methyl testosterone-triacetylglucuronic acid*. Methyl acetobromoglucuronate (700 mg.) and testosterone (500 mg.) were dissolved in benzene (25 ml.) and the solution shaken for 24 hr. with 1 g. of dry  $\text{Ag}_2\text{CO}_3$ . The debrominated solution was worked up as described under (I). The syrupy residue was dissolved in a little alcohol and the solution diluted with water. Crystallization occurred after a long time in the ice chest. The crystals were filtered off, washed with cold dilute alcohol and dried. After two recrystallizations from absolute alcohol and one from 96 % alcohol, 26 mg. of a substance were finally obtained which melted at  $186\text{--}189^\circ$  and crystallized in beautiful needles.

<sup>1</sup> All melting points were determined in a micro-melting point apparatus according to Kofler.

<sup>2</sup> The value for the rotation, which we have recently given in *Nature, Lond.*, **142**, 1036 (1938), was the result of a single determination and can only be considered as preliminary.

A crystalline mixture was obtained from the mother liquors, from which fractional crystallization from 50 % and finally from 70 % alcohol afforded a further 84 mg. (about 10.5 %) of pure substance.

Methyl testosterone-triacetylglucuronate crystallizes with 1 mol. of alcohol of crystallization, which is lost at 78°, the crystals becoming opaque. (Found: C, 62.49; H, 7.51 %.  $C_{32}H_{44}O_{11}$ ,  $C_2H_5OH$  requires C, 62.73; H, 7.75 %.) The compound had  $[\alpha]_D^{20} + 28.3^\circ$  ( $c = 0.006$  in  $CHCl_3$ ).

IV. *Methyl  $\alpha$ -oestradiol-3-benzoate-triacetylglucuronate*. Methyl acetobromoglucuronate (500 mg.) and the equivalent amount of  $\alpha$ -oestradiol-3-benzoate were dissolved in benzene (20 ml.), shaken for 24 hr. with 1 g. of dry  $Ag_2CO_3$ , and the product worked up as described in (I). The resultant syrup crystallized spontaneously when rubbed with a little absolute alcohol. Yield of crude product: 303 mg.; m.p. 165–175°.

After two recrystallizations from absolute alcohol, about 234 mg. (27 %) of pure substance were obtained, crystallizing in fine, felted needles, m.p. 188–191.5°. (Found: C, 65.73; H, 6.40 %.  $C_{38}H_{44}O_{12}$  requires C, 65.88; H, 6.51 %.)  $[\alpha]_D^{20} + 9.2^\circ$  ( $c = 0.0065$  in  $CHCl_3$ ).

223 mg. were dissolved in 30 ml. of methyl alcohol and 5 ml. of 0.33 N  $Ba(OH)_2$  and 5 ml. of water added to the solution. The Ba salt soon crystallized out in fine needles. The solution was boiled gently for 1 hr. under reflux (soda-lime tube) and, after cooling and diluting with water, the precipitate was filtered off and washed with hot water until the smell of methyl benzoate had disappeared. The salt was then dried in a vacuum over phosphorus pentoxide. Yield: 125 mg. (75 %).

V.  *$\alpha$ -Oestradiol-17-glucuronic acid*. 125 mg. of the above Ba salt were dissolved in 25 ml. of warm alcohol with the addition of 3.5 ml. of 0.1 N  $HCl$  and the warm solution was treated drop by drop with 0.1 N  $H_2SO_4$  until no further turbidity was produced. In this connexion it was observed that the precipitation of the Ba must take place hot, since in the cold an excess of acid precipitates the unchanged Ba salt. Nevertheless a slight excess of  $H_2SO_4$  is an advantage. The hot liquid was centrifuged and the residue extracted repeatedly with hot dilute alcohol. The combined, clear, acid centrifugates were carefully evaporated in a vacuum, after which crystallization soon occurred in the weakly alcoholic solution in the cold. After a long time in the ice chest, the precipitate was filtered off with suction and washed with ice-cold water containing methyl alcohol. The crystals were extremely thin, long, platelets arranged in rosettes. Yield: 87.5 mg. (80 %).

After recrystallizing twice from aqueous methyl alcohol, 56 mg. (about 52 %) were obtained, melting at 191–194.5° after drying in a high vacuum at 90° (the crystals sintered at 140–150°). (Found (ash-free): C, 61.02; H, 7.41 %.  $C_{24}H_{32}O_8$ , 1.5  $H_2O$  requires C, 60.60; H, 7.41 %.)

The substance was dissolved in alcohol, and the solution treated with 0.1 N  $HCl$  and a few drops of 0.1 N  $H_2SO_4$  in the cold. After 24 hr. in the ice chest the solution was scarcely turbid; it was clarified by filtration and part of the alcohol removed very carefully in a vacuum. Crystallization soon began in the form of small, irregular platelets. The gelatinous nature of the precipitate made filtration extremely slow. The pasty precipitate was again dissolved in a little alcohol and precipitated with water. The operation was repeated until the filtrates were free from acid. The substance was then dried, first in an ordinary vacuum and then in a high vacuum at 115–120°, when a faint tinge of yellow appeared. At this stage the carbon content was found to be too low and the hydrogen content too high; the substance was therefore again dried to constant weight in a high vacuum at

117°, when it lost a further 3.41% of water and the slight yellowing did not increase. (Found (ash-free): C, 64.19; H, 7.57%.  $C_{24}H_{32}O_8$  requires C, 64.25; H, 7.19%.)

VI. *Methyl oestrone-triacetylglucuronate*. Oestrone (142 mg.) and methyl acetobromoglucuronate (208 mg.) were dissolved in warm benzene (50 ml.) and the solution shaken overnight with 0.5 g. of dry  $Ag_2CO_3$ , after which the mixture was warmed for 30 min. on the water bath. The suspension was then centrifuged and the clear centrifugate worked up as usual. 52 mg. of substance were obtained, which was very sparingly soluble in cold absolute alcohol. After four recrystallizations from absolute alcohol-chloroform the m.p. was 225.5–228°. (Found: C, 63.28; H, 6.64%.  $C_{31}H_{38}O_{11}$  requires C, 63.45; H, 6.53%.)  $[\alpha]_D^{20} + 57.1^\circ$  ( $c = 0.0063$  in  $CHCl_3$ ).

VII. *Methyl cholesterol-triacetylglucuronate*. Methyl acetobromoglucuronate (150 mg.) and cholesterol (146 mg.) were dissolved in benzene (8 ml.) and shaken overnight with 300 mg. of  $Ag_2CO_3$ . The product was worked up as usual and the resultant syrup crystallized spontaneously when rubbed with alcohol. The yield of crude product was 40 mg. (15%). After three recrystallizations from absolute alcohol the substance melted at 162–164.5° after softening at about 152°. Yield of pure product: 18 mg. (about 7%).

The substance crystallizes in fine platelets with a mother of pearl lustre. (Found: C, 68.06; H, 8.88%.  $C_{40}H_{52}O_{10}$  requires C, 68.33; H, 8.89%.)

#### *Biological activities of products*

The following data may be given in the biological activities of the glucuronides.

I. A daily dose of 100  $\mu$ g. of methyl dehydroandrosterone-triacetylglucuronate (containing 47.6  $\mu$ g. of dehydroandrosterone) per capon, applied for 4 days dissolved in propylene glycol by smearing on the comb, was inactive.

II. A daily dose of 20  $\mu$ g. of dehydroandrosterone-glucuronic acid (containing 12.4  $\mu$ g. of dehydroandrosterone) per capon, applied for 4 days by smearing a solution in propylene glycol on the comb, was inactive. 77  $\mu$ g. (containing 47.7  $\mu$ g. of dehydroandrosterone) gave an average comb growth after 5 days of 14%.

III. A daily dose of 50  $\mu$ g. of methyl testosterone-triacetylglucuronate (containing 22  $\mu$ g. of testosterone), applied to each capon in the same way for 4 days, was inactive.

IV. 12  $\mu$ g. of methyl  $\alpha$ -oestradiol-3-benzoate-17-triacetylglucuronate (containing 6.5  $\mu$ g. of  $\alpha$ -oestradiol-3-benzoate) were inactive in castrated mice. 60  $\mu$ g. (containing 32.6  $\mu$ g. of  $\alpha$ -oestradiol-3-benzoate) produced oestrus when injected subcutaneously during 24 hr. in three lots of 20  $\mu$ g. each.

V. 12  $\mu$ g. of  $\alpha$ -oestradiol-17-glucuronic acid (containing 6.8  $\mu$ g. of  $\alpha$ -oestradiol) were inactive. 60  $\mu$ g. (containing 34.3  $\mu$ g. of  $\alpha$ -oestradiol), injected during 24 hr. in three lots of 20  $\mu$ g., produced oestrus.

VI. 30  $\mu$ g. of methyl oestrone-triacetylglucuronate (containing 13.8  $\mu$ g. of oestrone), injected subcutaneously in 24 hr. in three lots of 10  $\mu$ g., produced oestrus.

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# L. STUDIES ON THE PERMEABILITY OF ERYTHROCYTES

## VI. THE EFFECT OF REDUCING THE SALT CONTENT OF THE MEDIUM SURROUNDING THE CELL

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THE effect of diminishing the electrolyte content of the medium bathing the cell, keeping the osmotic pressure constant, is generally recognized as an injurious one, whereby the normal selective permeability of the plasma membrane is disturbed; as an example of this the work of McCutcheon & Lucke [1928], who found that in glucose solution the permeability of *Arbacia* eggs to water is doubled as compared with eggs in sea water, may be quoted. In earlier papers [Davson, 1934; 1936; 1937; Davson & Danielli, 1936; 1938] some of the conditions which determine the erythrocyte's normal impermeability to cations, and changes in these conditions which lead to a breakdown of this impermeability, have been described. The material presented here is an extension of the work to the special case of the effect of an absolute decrease in the quantity of electrolytes in the suspension medium.

Joel [1915] has shown that if the erythrocytes of the ox are washed repeatedly with sugar solution and then suspended in the same medium, the latter shows an increase in electrical conductivity with time which is due, as he supposed, to the passage of salts from the cells; however, this treatment is drastic and there is no evidence that the changes observed were reversible and were not due to haemolysis. Jacobs & Parpart [1933], working under less extreme conditions, found changes in the fragility of the same erythrocytes on exposure to hypotonic solutions of non-electrolytes which they interpreted as being due partly to anionic exchanges and partly to the loss of cations from the cells. Maizels [1935] found, by direct chemical analyses, that the human erythrocyte loses K in glucose solution in a reproducible manner, the effect increasing with temperature.

The results of Joel and of Jacobs & Parpart are essentially indirect and, whilst the constructions placed upon them appear quite probable, experience has shown [Davson, 1936; 1937; Davson & Ponder, 1938] that indirect measurements as in conductivity and fragility studies are of questionable value in regard even to qualitative estimates of changes in cation permeability, whilst quantitative measurements are virtually excluded. Thus the results of Maizels remain as certain facts, inadequate in themselves, however, to allow of any explanation of the phenomenon of the induced cation permeability due to the reduction in the electrolyte content of the medium. Before entering into the experimental part of this paper, possible mechanisms for the cation permeability and the kinetics of the loss of K may be briefly touched upon.

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If an erythrocyte is placed in a non-electrolyte solution there will, as Jacobs & Parpart [1933] have shown, be a tendency for the  $\text{Cl}^-$  and  $\text{HCO}_3^-$  to leave the cell; this loss of anions will be replaced by  $\text{OH}^-$  from the non-electrolyte medium. Thus, owing to the rapid shift of anions across the membrane, there will be a difference of  $p\text{H}$  (which may amount to three units if the dilution of the blood is great enough), the cells being alkaline in respect to the suspension medium. What seems not to have been realized hitherto is that in consequence of this  $p\text{H}$  difference there will be a large potential difference between the inside and the outside of the cell, the latter being negative in respect to the former. The magnitude of this P.D. will be given by the formula:

$$E = RT/F \ln [H_0]/[H_i],$$

where  $[H_0]$ ,  $[H_i]$  are the respective H ion concentrations inside and outside the cell, and thus may be of the order of 150 mV. As a result it would not be surprising if positive ions, should they come under the influence of this P.D., were repelled from the inside to the outside of the cell so that the normal restraint on these ions, which inhibits their migration, is overcome. Thus the phenomenon of cation permeability might reasonably be expected on suspending erythrocytes in a non-electrolyte medium. As K escapes from the cell it will be accompanied by  $\text{Cl}^-$  and  $\text{HCO}_3^-$  and, to a lesser extent, by  $\text{OH}^-$ ; as a result of the migration of the  $\text{HCO}_3^-$  and  $\text{OH}^-$  ions the difference of  $p\text{H}$  tends to be reversed, and also the sign of the P.D., as the loss of K proceeds. If the cation permeability is due entirely to the acceleration of the ions down a potential gradient, then it must cease as soon as the difference in  $p\text{H}$  is neutralized; furthermore, the addition of salts at any time during the escape of K, in so far as it reverses the P.D. caused by the shift of anions, should inhibit the further loss of K.

If, however, the cause of the cation permeability is not the electrostatic acceleration of the ions but is due to the potential difference acting directly on the membrane, then the inhibition of the cation permeability due to the levelling out of the P.D. is only to be expected if the change in the membrane is a reversible one. If, further, it can be shown that addition of salts inhibits the migration of K after it has begun, and also that the latter process does *not* cease when the P.D. is neutralized or reversed in sign, it follows that the cation permeability is not due to the P.D. at all, and its cause must be sought in an actual change in the membrane due to the reduction in the salt content of the medium in contact with its exterior. The experimental work to be described may thus be considered in respect to these three general theories and it will be seen that by a process of exclusion one must adopt the last-mentioned in explaining the permeability to cations in non-electrolyte media.

The rate of loss of K will be given by the simple unimolecular equation:

$$\frac{dK_i}{dt} = k (K_i - K_0 - \beta),$$

where  $k$  is the permeability constant,<sup>1</sup>  $A$  the area of the cells,  $K_i$  and  $K_0$  the concentrations of K inside and outside the cells respectively and the constant  $\beta$  equals the difference between the concentrations of K inside and outside the

<sup>1</sup> The use of a permeability constant for a single ion is not strictly correct, since the rate of permeation of a given ion will be a function of two probabilities, viz. those that a positive ion and a negative ion will pass the membrane at any given moment. However, in the case of the erythrocyte, which is so freely permeable to  $\text{Cl}^-$  and  $\text{HCO}_3^-$ , the use of a single constant for  $\text{K}^+$  is probably justified, at least for the purposes of this paper. It will be seen that the experimental results deviate so greatly from the equation that this consideration is a minor one compared with the auto-inhibitory effect of the escape of K *per se*.

cells at equilibrium and is introduced into the equation to satisfy the condition that  $dK_i/dt$  equals zero when the K concentration inside the cell has dropped to its equilibrium value, which will be determined by the Donnan equilibrium, and will be greater than the concentration in the suspension medium; its absolute value will be small compared with the initial value of  $K_i$  owing to the large volume of suspension medium. *The equation assumes that the conditions determining the escape of K are unaffected by this escape.*

Now to a first approximation  $K_i$ , the concentration of K inside the cells, will be constant, since the loss of K plus its accompanying anions will result in a decrease in the osmotic pressure inside the cell causing the passage of  $H_2O$  into the suspension medium.<sup>1</sup> Under the conditions of the experiments  $K_0$  may also be treated as a constant, and, owing to the disk shape of the cells,  $A$  will be approximately constant. Hence the equation becomes:

$$dK_i/dt = \text{Constant},$$

so that the loss of K should proceed linearly with time, and any gross deviation from linearity should be ascribed to a variation in  $k$ , the permeability constant, i.e. to a change in the properties of the membrane or the p.d. across it due to the loss of K.

#### EXPERIMENTAL

The non-electrolyte solution used was 0.32 *M* glucose, in glass-distilled water, for all species except human for which 0.32 *M* sucrose was used, since human erythrocytes are comparatively permeable to glucose. The whole blood was added to the non-electrolyte solution, contained in a centrifuge tube immersed in a thermostat at 25°: the ratio of the volumes of blood to suspension medium was 1:10. After the appropriate interval the suspension was centrifuged, the supernatant fluid was removed and the K or Na content of the cells determined as described earlier [Davson, 1934; 1937] using the method of Kramer [1920] for K and that of Barber & Kolthoff [1928] for Na. If the cells contained only a little K, as in the case of the cat, the dissolved ash was not made up to a definite volume from which aliquots could be taken but was evaporated to dryness in a silica beaker and the whole of the K in it determined; this procedure was also used for all Na determinations. The errors arising out of all steps in the procedure rarely amounted to more than 1%. As controls, cells suspended in non-electrolyte medium and immediately centrifuged down were used so that losses due to the centrifuging of the cells *per se* were discounted. pH changes in the suspension medium were determined colorimetrically.

#### RESULTS

In Table I the variation in the K contents of the erythrocytes of the man, guinea-pig, rat, pig and rabbit with time of suspension in a non-electrolyte medium are shown; figures for three individuals of each species are presented to give some idea of the variability in the magnitude of the changes. The results are expressed as percentages of the control value. In Table II similar figures are given for the K and Na contents of the erythrocytes of the ox and cat: these

<sup>1</sup> Since about one-third of the K in the cells is in the form of a polyvalent haemoglobin salt,  $K_nHb$ , there will be an excess of osmotically active cations over anions, so that the loss of a pair of ions ( $K + Cl^-$ ) or ( $K + HCO_3^-$ ) reduces the osmotic pressure inside the cells by a greater amount than the fractional decrease in the K content. Hence, strictly speaking, the concentration of K should rise in the cells, although the amount per cell falls. This effect is compensated, however, by the increasing acidity inside the cell which reduces the amount of  $K_nHb$ .



Table I. *The variation of the K content of the erythrocytes of different species with time of suspension in a non-electrolyte medium. Temp. 25°*

Time	Man			Guinea-pig			Rat			Pig			Rabbit		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Control	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 hr.	74	80	82	84	91	98	95	93	92	99	—	99	98	100	101
2 hr.	57	69	69	76	79	94	89	87	86	99	99	99	98	99	100
3 hr.	52	60	59	69	74	93	86	83	—	93	—	99	98	99	96
6 hr.	—	—	—	—	—	—	—	—	—	—	96	98	—	—	—

Table II. *The variation of the K and Na contents of the erythrocytes of the ox and cat with time of suspension in a non-electrolyte medium. Temp. 25°*

Time	Ox						Cat					
	I		II		III		I		II		III	
	K	Na	K	Na	K	Na	K	Na	K	Na	K	Na
Control	100	100	100	100	100	100	100	100	100	100	100	100
1 hr.	95	98	95	96	100	100	99	98	94	97	95	92
2 hr.	95	98	92	91	97	98	92	85	88	94	87	71
3 hr.	92	95	91	90	93	96	89	66	87	90	78	34
5 hr.	70	77	—	—	—	—	—	—	—	—	—	—

species differ from those in Table I in that their erythrocytes contain more Na than K. From Table I it is evident that the erythrocytes of the man, guinea-pig and rat show a well-defined permeability to K when suspended in a non-electrolyte medium; the cells of the pig and rabbit, however, are sharply differentiated from the former group in that the loss of K in the first 3 hr. is very small and of the order of 1–2%. This species difference will be taken up later.

With regard to the results in Table II it may be remarked that the cells of the ox and cat show irregularities in their behaviour which were not encountered with the species in Table I. Thus in Exp. 1 of the ox there is a loss of 5% in the K content during the first hour, no loss during the second hour, a loss of 3% during the third, and of 22% during the last 2 hr.; the same anomalous course was followed by the Na values; again in Exp. 1 of the cat we have a loss of 1% of the K during the first hour, 8% during the second hour and 3% during the third hour. Another sort of irregularity occurs most markedly with the cat erythrocytes; this is in respect of the relative rates of loss of Na and K. It is generally realized that although K has a larger atomic weight than Na, the size of the K ion in aqueous solution is smaller than that of the Na ion in virtue of the greater hydration of the latter; consequently, if size determines the relative rates of migration of the two ions across the membrane, K should penetrate more rapidly than Na. This appears to be the case with the ox, and on occasions with the cat, but Exps. 1 and 3 for the cat show a much greater percentage loss of Na than of K during the intervals 1, 2 and 3 hr. That these irregularities can be due to experimental errors is quite out of the question, so that it would appear that under certain conditions Na may penetrate a membrane more rapidly than K, unless, of course, the equilibrium conditions determining the cessation of the permeability process are more complicated than would generally be expected; thus if the K in the cells is, at the outset, much nearer its equilibrium value than the Na, then comparisons between the losses of K and Na in given times would not necessarily lead to the determination of comparative permeability constants. The acceleration in the rate of loss of cations with time mentioned above will be discussed more fully later.

To return to the behaviour of some of the species in Table I, the K contents of the human, guinea-pig and rat cells are plotted against time of suspension in a non-electrolyte medium in Fig. 1; it will be seen from Table I that the curves are probably typical of the species, the rate of loss being in the order: man > guinea-pig > rat. The curves show deviations from linearity, the deviation being more pronounced and showing itself sooner the greater the initial rate of

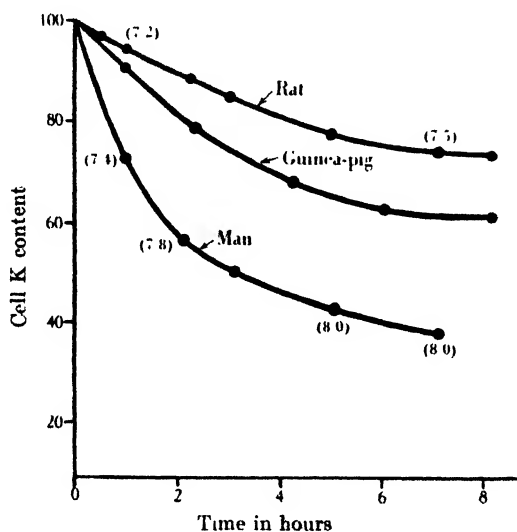


Fig. 1. Loss of K from the erythrocytes of the rat, guinea-pig and man in non-electrolyte solution at 25°. Ordinates: cell K content as a percentage of the control value. Abscissae: time of suspension in the non-electrolyte medium in hours. Figures in parentheses indicate the pH of the suspension medium after centrifuging down the cells.

loss. Furthermore, the curves tend to different stationary states; thus the rat cells tend to a value of 72.5, the guinea-pig to one of 60 and the human to 35 or less, these figures being, as before, percentages of the initial cell K contents. The figures near points on the curves are the values for the pH of the suspension medium after centrifuging and it is clear that the change over from an acid medium to an alkaline one mentioned in the introduction certainly occurs. However, the loss of K does not cease as soon as the medium becomes alkaline.

The reversibility of the induced permeability to K is brought out by the results of Table III and Fig. 2. In Table III, row (a) gives the loss of K during 1 hr. of suspension in the non-electrolyte medium followed by 2 hr. of suspension in the same medium to which NaCl had been added to bring the concentration of the latter up to 0.10 M. Row (b) gives the loss of K during 1 hr. of suspension in the non-electrolyte medium, and row (c) the loss during 2 hr. suspension in non-electrolyte solution plus NaCl. If the process is reversible (b) plus (c) should be equal to or greater than (a), and this is the case within the limits of experimental error. In Fig. 2, the curve AB gives the loss of human cells with time in sucrose solution; AC represents the loss in a sucrose plus NaCl solution (0.10 M with respect to NaCl). At the points X NaCl was added as before and the lines XX' show the loss of K subsequent to the addition of NaCl. The approximate parallelism of the lines again indicates reversibility, even after 2 hr. in the non-electrolyte medium alone. The slight loss of K from the cells in sucrose plus

Table III. *Experiments designed to show the reversibility of the induced permeability to K. (N.E. is abbreviation for non-electrolyte solution)*

Treatment	% loss of K		
	Man	Guinea-pig	Rat
(a) 1 hr. in N.E.; 2 hr. in N.E. + NaCl	20	23	13
(b) 1 hr. in N.E.	20	24	13
(c) 2 hr. in N.E. + NaCl	2	3	0
(b) + (c)	22	27	13

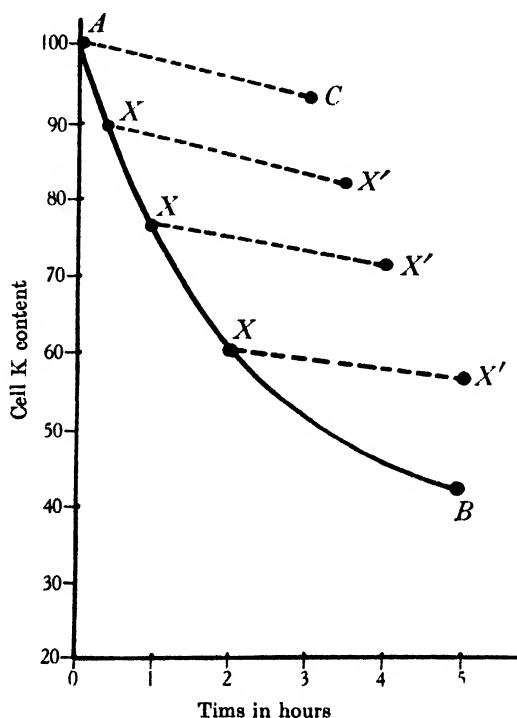


Fig. 2. Curves demonstrating the reversibility of the induced permeability to K. Temp. 25°.

NaCl indicated by the line AC is not due to the presence of sucrose since it occurs in isotonic NaCl alone [Maizels, 1935; Davson, 1937].

In Fig. 3 the loss of K from the human erythrocyte in 2 hr. is shown as a function of the electrolyte content of the medium and it is seen that the effect of diminishing the electrolyte content of the medium begins when the former has been reduced to less than 0.09 *M*. The extreme sensitivity of the rate of loss to salt content of the suspension medium at values of the latter below 0.016 *M* is another interesting point.

From the curves in Fig. 1, the coincidence of the retardation of the rate of loss of K with the increasing alkalinity of the suspension medium might lead one to suppose that the cause of this retardation was the alkalinity; this, however, would be in contradiction to Maizels' results which showed an increased rate of loss in an alkaline medium, although too much weight must not be placed on these results since the alkalinity was produced by addition of NaOH to the medium giving an initial reaction of the medium of about pH 10 which in itself is sufficient

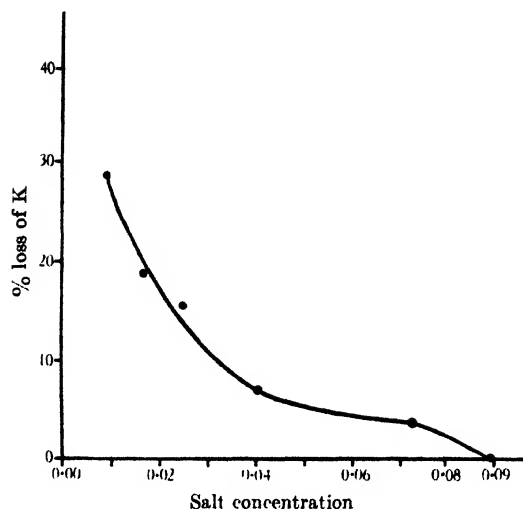


Fig. 3. Variation in the rate of loss of K from human erythrocytes with salt content of the suspension medium. Ordinates: percentage loss of K in 2 hr. Abscissae: concentration of NaCl in the suspension medium in moles/litre. Temp. 25°.

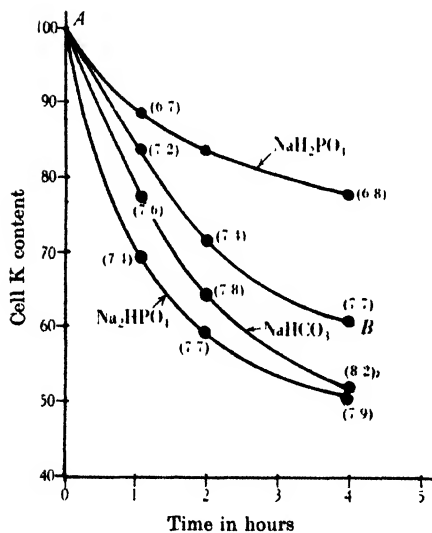


Fig. 4.

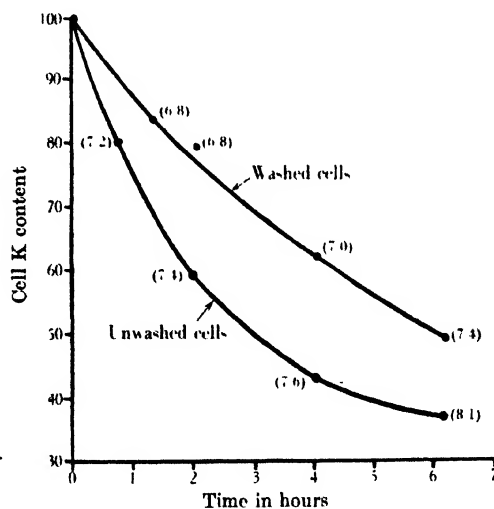


Fig. 5.

Fig. 4. The effect of the addition of acid and alkaline buffers to the non-electrolyte medium on the loss of K from human cells. AB is the control curve for non-electrolyte medium plus NaCl. Temp. 25°.

Fig. 5. Comparison of the losses of K from washed and unwashed human cells in non-electrolyte media. Temp. 25°.

to produce irreversible damage. In Fig. 4 is shown the effect of the addition of the buffers  $\text{NaH}_2\text{PO}_4$ ,  $\text{NaHCO}_3$  and  $\text{Na}_2\text{HPO}_4$  to the non-electrolyte medium on the loss of K from human cells. The control curve, AB, represents the loss of K in sucrose solution to which NaCl had been added to make the total ionic con-

The results shown in Table IV may now be briefly enumerated. Exp. 1 shows that the erythrocytes of the rabbit maintain their virtual impermeability to K in glucose solution for as long as 7 hr. of suspension; there is indeed a loss of 4% by the end of this time but the figures show that it is distributed evenly over the whole period. Washing the cells of the rabbit twice with glucose before suspension in glucose solution for a definite time causes a fair degree of permeability to K (Exp. 2). Addition of copper to the non-electrolyte medium causes a rapid sedimentation of the rabbit cells but does not produce any marked permeability to K.

## DISCUSSION

The theory that the permeability of the erythrocyte to cations, induced by the diminution of the electrolyte content of the suspension medium, is due simply to an electrostatic repulsion of K and Na by the potential gradient across the membrane must be abandoned, since the loss of cations does not cease when the suspension medium becomes more alkaline than the contents of the cell (in virtue of the buffering power of haemoglobin very little change in the pH of the inside of the cell will occur during the escape of K; any change which does occur will be to make the contents more acid). As the P.D. considered here is a thermodynamic one, depending on the relative concentrations of  $\text{Cl}^-$  and  $\text{HCO}_3^-$  in the cells and suspension medium, only small and irregular variations between species should occur in it. However, it is found that the rabbit and pig cells show little tendency to lose K under conditions where human cells lose as much as 70% in 7-8 hr. This again presents a difficulty in the way of interpreting the permeability as an electrostatic phenomenon, although it may be overcome by the assumption that the normal restraints on the migration of cations vary in strength among the species, being greatest with the rabbit and pig and least with the human cells. This assumption is not in accord, however, with previous work on species characteristics [Davson, 1937]. Passing, then, to the next possible mechanism, viz. that the P.D. acts on the membrane, possibly by a re-orientation of the dipole constituents of the membrane, then the loss of K must only be expected to cease when the P.D. is neutralized provided that the change in the membrane is reversible. Now it has been shown that the induced permeability to K is indeed reversible by addition of salts, so that it would, on this theory, be necessary that the permeability to K should cease as soon as the P.D. were neutralized. We must therefore reject the P.D. as a significant factor in causing the permeability to cations; hence the cause of the permeability must be sought in a reversible change in the membrane due to the decrease in the salt content *per se*, apart from any potential effects due to ionic exchanges across this membrane.

Attention should be drawn here to a correlation between the magnitude of the induced permeability to cations and the rate of sedimentation of the erythrocytes in the non-electrolyte solutions. The erythrocytes of all species generally sediment more rapidly when suspended in a non-electrolyte solution than in isotonic NaCl, but this difference is far more pronounced in the case of the human, guinea-pig, rat, ox and cat cells than with the rabbit and pig cells; in fact individual samples of pig blood have been obtained which showed no evidence of settling, when added to glucose solution, after an hour, whereas if human or guinea-pig blood is treated in the same way the cells drop to the bottom of the tube within 5 min. or less. The reason for the rapid sedimentation is, of course, the agglutination; the suspensions which sediment rapidly contain large aggregates of cells whereas a suspension of pig blood is found to contain only discrete cells.<sup>1</sup> Washing the rabbit cells, which normally show very little permeability to cations in a non-electrolyte medium, twice with glucose solution and then suspending them in a further quantity causes them to lose K fairly readily, and at the same time marked agglutination is observed. Thus there

<sup>1</sup> This correlation is not perfect; thus one specimen of rat blood gave no sign of sedimentation and yet lost K at a rate typical for this species. Similarly, a specimen of rabbit blood sedimented fairly rapidly with no loss of K; in this case, nevertheless, there was no observable agglutination. No specimen of human, guinea-pig, or cat blood has, however, been found which did not show marked agglutination.

seems to be some change in the surface of the erythrocyte, produced by the reduction of the electrolyte content of the medium, which allows agglutination to occur and at the same time induces a permeability to cations. That the agglutination itself does not cause the cation permeability but is only symptomatic of the changes which do produce it is shown by the fact that addition of Cu to the non-electrolyte medium, which causes rabbit cells to agglutinate to a tremendous extent, does not cause any marked loss of K; further, addition of acid phosphate to the medium decreases the rate at which K escapes from human cells whilst it increases the degree of agglutination. The theory that a change in the surface of the erythrocyte is responsible for the cation permeability would be in accord with the apparently anomalous behaviour of the ox cells: here we had an acceleration of the rate of loss as the time of suspension increased, and this could be accounted for by assuming that the change in state of the surface of the erythrocytes of this species takes time, so that during the first hour or so the cells will not be very permeable whereas later, when the change becomes more pronounced, the permeability increases.

To determine the nature of this surface change presents some difficulties. The effect of a reduction in the salt content of the medium on such systems as protein sols or thin films of lipoids has been studied and has been described as a "shift of the isoelectric point" [Michaelis & Rona, 1919] or a change of the "surface pH" [Danielli, 1937], and these descriptions agree in so far as the changes expected will be those occasioned by a shift of the pH of the medium to the acid side, if the dissociating groups in the systems are on the alkaline side of their isoelectric points. As the ionizing groups in the erythrocyte membrane are quite definitely on the alkaline side of their isoelectric point [see e.g. Abramson, 1934], if the phenomenon of the induced permeability to cations has any similarity with the changes described by Michaelis & Rona and by Danielli, then it would be expected that it would be inhibited by alkaline and accelerated by acid buffers, yet we have seen that the reverse is true; acid phosphate reduces the rate of loss of K far more than an equivalent quantity of a neutral salt such as NaCl, whilst alkaline phosphate or bicarbonate does not reduce the rate of loss as much as NaCl does. The change in the surface of the erythrocyte is, then, not one which could be simply ascribed to a change in the ionization of its acid groups, and it is interesting to note, if the correlation between the agglutination of the cells and their induced permeability to K means anything, that Abramson [1934] has shown that the charge on the surface of the erythrocyte, measured electrocataphoretically, is not the determining factor in rouleau formation and therefore, presumably, in agglutination. Further investigation of this point must be on a system which allows changes in the salt content of the medium being made independently of changes in the pH. With the intact erythrocyte this is impossible owing to the exchanges of anions. Such a system might be given by a spread film of the extracted lipoids from the erythrocyte [Gorter & Grendel, 1925] and work on this is under way [Danielli & Davson]; however, it may well be that the permeability to cations is a result of an asymmetry of the membrane, since the concentrations of salts inside and outside are, under the conditions of suspension in a non-electrolyte medium, widely different, so that studies on films may not throw light on the problem.

To return to the actual experimental data, there is a final point which requires attention, namely the falling off in the rate of loss of K with time, which occurs at quite an early stage in the process. The equation derived to suit the conditions of the experiments obviously does not fit the observed data, and it seems, in view of the large deviation from linearity, that the discrepancy is not

due to any of the approximations introduced in the derivation, but rather to the "permeability constant" being a function of the loss of K at any given moment. The alkalinity of the suspension medium caused by the migration of K is not the cause of this auto-inhibition since alkalinity favours the loss of K; similarly the increased salt content of the medium can have little influence owing to the large volume of the latter. A possible factor is the p.d. which will be built up by the migrating  $\text{HCO}_3^-$ ; as the loss of K proceeds, the inside of the cells will become acid whilst the suspension medium becomes alkaline, and this difference in reaction will be reflected in a difference in potential, and this latter may eventually become great enough to inhibit the further loss of K. Support is given to this view by the curves of Fig. 5, in which the losses of K from cells, out of which most of the  $\text{HCO}_3^-$  has been washed, are compared with those from cells with their normal contents. It was seen that the washed cells showed only a very little inhibition of the loss of K after 6 hr. of suspension whilst the unwashed cells showed marked inhibition after 2 hr. suspension. This difference in behaviour may be attributed to the difference in the  $\text{HCO}_3^-$  content of the cells; the passage of a given quantity of K will be followed by a greater pH difference when this K is accompanied by  $\text{HCO}_3^-$  than when it is accompanied by Cl<sup>-</sup>. It was noted that the pH values for the suspension media agreed with this view. Thus although the p.d. due to an excess of  $\text{OH}^-$  in the cells over that in the suspension medium is not the chief factor in causing the migration of K, it may be of importance in determining its cessation. The slower the initial rate of loss, i.e. the greater the obstacle offered by the membrane to the passage of K, the smaller will be the p.d. required to inhibit the loss; hence the loss of K from the rat cells will be expected to cease at a smaller external pH than that from human cells, and this is so.

Two practical points following from this work may be touched upon. Many studies of the permeability of erythrocytes to non-electrolytes are made by adding a small quantity of blood to a large volume of the pure non-electrolyte solution, and using the time required for haemolysis as an index to the rate of penetration of the non-electrolyte [*vide e.g. Jacobs et al. 1935*]. The customary dilution of the blood in the non-electrolyte medium is 1 in 500, and it is quite clear from the present work that even at a much lower dilution (1 in 10) the erythrocytes of many species are in a definitely abnormal state, so that the results of this type of work, interesting and important as they are, may nevertheless not represent the behaviour of cells in their normal environment. *Jacobs et al. [1937]* have indeed studied the influence of salts on the permeability of ox erythrocytes to glycerol, and have found changes which, however, can be at least partly, if not wholly, ascribed to changes in the volume of the cells due to anionic exchanges. It would be interesting if this work were extended to the erythrocytes of species which show cation permeability in a non-electrolyte medium more markedly, e.g. human and guinea-pig cells, since the demonstration of a normal behaviour in respect to the permeability to non-electrolytes and an abnormal behaviour regarding cation permeability might lead to important conclusions relating to the structure of the erythrocyte membrane.

A more disturbing point is the effect of the escape of K on the rate of haemolysis when this is being used as an index to the rate of penetration of a non-electrolyte. From the curve in Fig. 3 it follows that with a dilution of blood of 1 : 500 the rate of loss of K from the erythrocytes of some species may be many times greater than that described here; as a result of this loss of K the amount of non-electrolyte which must penetrate a cell to cause it to burst will be greater, so that the time required for haemolysis will be no true index to the rate



of penetration of the non-electrolyte. This will be especially true in respect to the determination of temperature coefficients, since the effect of a rise in temperature will be two-fold: (a) an increase in the rate of penetration of the non-electrolyte, and (b) an increased escape of K. These effects will be antagonistic, and it is interesting to note that Jacobs *et al.* [1935] find with certain species an actual decrease in the rate of haemolysis in glycerol solutions on raising the temperature, and this may be due to the fact that the influence of (b) outweighs that of (a). Probably this consideration does not affect the main conclusions drawn by Jacobs *et al.* from their measurements of temperature coefficients, but it would be a serious factor in any calculations of the kinetics of penetration in which values of the  $Q_{10}$  determined under these conditions are included.

#### SUMMARY

The permeability of the erythrocytes of several species to cations, on suspending them in a non-electrolyte medium, has been investigated. The cells of the man, guinea-pig and rat are permeable to K, and those of the ox and cat to both Na and K, under these conditions; the latter species show irregularities in that there may be an acceleration of the rate of loss of cations with time, and in that Na may sometimes penetrate more rapidly than K. The cells of the man, guinea-pig and rat give smooth curves on plotting their K contents against time of suspension; however, there is a falling off with time in the rate of loss of K, so that a stationary state is reached at times remote from the equilibrium position. This auto-inhibition is ascribed tentatively to the building up of a potential gradient which eventually inhibits the further migration of K. The erythrocytes of the rabbit and pig show little tendency to lose K under the same conditions, the loss after 7 hr. of suspension may be as low as 4% compared with a loss of nearly 70% with human cells.

The change in the membrane causing the permeability is a reversible one in that the escape of K may be inhibited after it has begun by the addition of NaCl to the non-electrolyte medium.

Human cells are very sensitive to salt concentration of the suspension medium when this is less than 0.02 *M*; at a concentration of 0.09 *M* the non-electrolyte effect ceases.

Alkaline buffers added to the suspension medium reduce the rate of loss of K to a smaller extent than an equivalent quantity of NaCl; acid buffers have the reverse effect.

There is an apparent correlation between the tendency of the erythrocytes of different species to agglutinate in a non-electrolyte medium and the induced permeability to K; the agglutination itself is not the cause of the permeability to K, but may be symptomatic of a change in the surface of the cell which allows cations to cross the membrane.

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# LI. THE ZINC CONTENT OF EPIDERMAL STRUCTURES IN BERIBERI

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THE author [1938] has shown by calculation from the known Zn contents of a large variety of foodstuffs that the average amount of Zn ingested per day in a balanced diet is about 12 mg. and that this figure agrees with the average amount of Zn found by several workers to be eliminated daily in the faeces and urine of normal individuals. The author has also drawn attention to the fact that each of the three foods, sea fish, cooked polished rice and cabbage, which form the bulk of the diet of the poorest class of Chinese in South China, contains less than 6 mg. Zn per kg. of food as eaten. Since the amount of food consumed in a day by a person existing on such a restricted diet would not appreciably exceed 1 kg., it may be assumed that this represents the approximate daily Zn intake. Small additions of other foodstuffs richer in Zn will not materially augment this figure.

At this point it was realized that apart from containing little Zn, the three foodstuffs mentioned were also poor in aneurin. An examination of the available published data revealed strong evidence of a positive correlation between the Zn and aneurin contents of foodstuffs as shown in Table I and Fig. 1. For

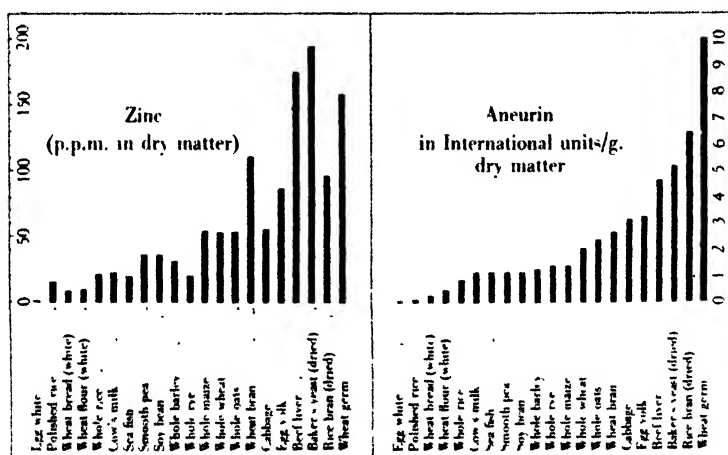


Fig. 1. Zinc and aneurin contents of foodstuffs.

this reason a diet supplying inadequate aneurin should also supply subnormal amounts of Zn and it would be reasonable to suppose therefore that sufferers from beriberi would show a reduction in the Zn content of their tissues.

Table I

Foodstuffs	Moisture† content %	Zn content*			
		Fresh matter			Dry matter p.p.m.
		Range p.p.m.	Mean p.p.m.	No. of observations	
Egg white	87.5	Nil-trace	Nil-trace	(4)	Nil-trace
Polished rice	15	9.7 - 14.5	13	(7)	15
Wheat bread (white)	33	4.1 - 8.0	5	(3)	8
Wheat flour (white)	20	6.5 - 8.0	7	(3)	9
Whole rice	15	14.65- 22.4	18	(3)	22
Cow's milk	86.5	2.07- 5.6	3.1	> (30)	23
Sea fish	80	0.3 - 8.0	4	(6)	20
Smooth pea	10	28.0 - 40.4	33	(4)	36
Soy bean	8	27.3, 38.0	33	(2)	36
Whole barley	15	—	27	(1)	31
Whole rye	15	—	17	(1)	20
Whole maize	54	—	25	(1)	55
Whole wheat	15	22.1 - 84.8	45	(5)	53
Whole oats	15	31.71- 77.5	45	(6)	53
Wheat bran	10	74.0 -139.2	100	(7)	111
Cabbage	94	2.6 - 3.7	3	(3)	55
Egg yolk	53	23.0 - 56.7	40	(8)	86
Beef liver	69	34.5 - 84.0	54	(7)	175
Baker's yeast (dried)	—	36.4 -305	194	(4)	194
Rice bran (dried)	—	67.4, 70	69	(2)	69
Wheat germ	10	140, 145	142	(2)	158

Foodstuffs	Moisture† content %	Aneurin content‡			
		Fresh matter			Dry matter I.U./g.
		Range I.U./g.	Mean I.U./g.	No. of observations	
Egg white	87.5	Nil-trace	Nil-trace	(2)	—
Polished rice	15	0.0 - 0.30	0.15	(4)	0.18
Wheat bread (white)	33	0.07 - 0.30	0.18	(18)	0.27
Wheat flour (white)	20	0.0 - 1.2	0.38	(11)	0.47
Whole rice	15	0.2 - 1.2	0.68	(21)	0.80
Cow's milk	86.5	0.06 - 0.25	0.15	(16)	1.1
Sea fish	80	0.0 - 1.0	0.22	(17)	1.1
Smooth pea	10	0.4 - 1.3	1.0	(4)	1.1
Soy bean	8	0.45 - 1.5	1.0	(8)	1.1
Whole barley	15	1.0 - 1.2	1.1	(4)	1.2
Whole rye	15	1.0 - 1.36	1.1	(3)	1.3
Whole maize	54	0.05 - 1.0	0.59	(5)	1.3
Whole wheat	15	0.75 - 3.40	1.7	(26)	2.0
Whole oats	15	1.1 - 3.25	1.9	(3)	2.3
Wheat bran	10	1.3 - 3.6	2.3	(6)	2.6
Cabbage	94	0.076- 0.25	0.19	(7)	3.1
Egg yolk	53	1.4 - 1.6	1.5	(2)	3.2
Beef liver	69	1.2 - 1.6	1.4	(4)	4.6
Baker's yeast (dried)	—	2.4 -10.0	5.1	(14)	5.1
Rice bran (dried)	—	4.2 - 7.6	5.8	(3)	6.4
Wheat germ	10	6.5 -22.0	90	(20)	10.0

\* Derived from various sources quoted by Eggleton [1938].

† Derived from Read *et al.* [1937].

‡ Derived mainly from sources quoted by Fixsen & Roscoe [1938] but including also some values given by Pyke [1937].

Since epidermal structures are obtainable with equal ease from the living as from the dead and since they show little post-mortem change compared with internal viscera, it was decided to test the suggested correlation by investigating the Zn content of epidermal structures of patients with beriberi and normal individuals.

It is apparent that an abnormally low content of Zn in the epidermal structures might result also from low intake of food of normal Zn content. This starvation factor in the case of patients with beriberi cannot be ignored, but the correlation between the Zn and aneurin contents of foodstuffs shows that reduced epidermal Zn in beriberi is not wholly due to reduced food intake.

### Methods

The samples were obtained partly from autopsies and partly from living persons, choice of material being governed by the need for keeping the comparison as unbiased and clear-cut as possible. It should be mentioned that in one case beriberi was complicated by amoebic dysentery with perforation which caused death. In another, a survival case, the beriberi had developed after cholera. The normal subjects were chosen from healthy living persons or from healthy accident cases or suicides showing no gross pathological changes. With three exceptions all samples were taken under the author's supervision.

The method of analysis used was essentially that described by Sylvester & Hughes [1936], which involves dry-ashing the clean, ether-extracted material, dissolving in HCl and extracting with dithizone at a specified pH. The Zn, after isolation from the dithizone, was estimated by the volumetric method of Lang [1929; 1933]. Several modifications making for increased accuracy and speed have been introduced and will be described elsewhere.

### Results

The analytical results given in Table II show that the Zn content of the hair, nails and skin of patients with beriberi is about half that of those not suffering from the disease and that the ash contents of the nails and skin appear

Table II. *Zn and ash contents of epidermal structures in beriberi*

	Zinc content (p.p.m. of fat-free dry material)		Non-beriberi	
	Range	Mean	Range	Mean
Head hair (pigmented)	110-229	173 (6)	84-444	225 (11)
Head hair (unpigmented)	145	—	198, 222	210 (2)
Beard hair	—	—	130, 167	149 (2)
Pubic hair	45-115	83 (4)	71-342	197 (8)
Finger nails	22-184	88 (13)	121-260	195 (13)
Toe nails	31-153	90 (6)	96-340	198 (11)
Skin (whole)	6-21	13 (7)	12-55	26 (8)
Epidermis	—	—	97	—
Ash content (% of fat-free dry material)				
Head hair (pigmented)	0.16-0.39	0.28 (7)	0.23-0.73	0.45 (10)
Head hair (unpigmented)	0.095	—	0.53, 0.72	0.63 (2)
	0.091	—		
Beard hair	—	—	0.31, 1.03	0.67 (2)
Pubic hair	0.18-0.70	0.51 (6)	0.10-0.75	0.40 (7)
Finger nails	0.22-3.35	1.18 (14)	0.07-0.56	0.30 (10)
Toe nails	0.42-1.53	0.68 (7)	0.15-0.72	0.39 (8)
Skin (whole)	2.33-3.90	2.92 (7)	1.37-2.56	2.08 (5)
Epidermis	—	—	0.12	—

Figures in brackets indicate number of subjects examined.

to be increased in beriberi. Why the ash content should be increased is not clear unless it be the result of diminished keratin formation arising from diminished protein intake. The determination of the ash in the small quantities of material

used is not so accurate as the determination of the Zn, so that less emphasis can be placed on the figures obtained, but the figures for Zn are clear-cut and leave no room for doubt.

### Discussion

The idea of an association between the mineral content of a foodstuff and its aneurin content is not new. Schaumann [1908] pointed out that diets producing peripheral neuritis were invariably poor in phosphorus. In the erroneous belief that nucleic acid was the active principle he suggested that the P content of antineuritic substances was a direct measure of potency. Haag & Palmer [1928] have also drawn attention to the fact that some symptoms of aneurin deficiency are similar to those of Ca and P deficiency. Kilbourne [1910] has also pointed out that the potassium of rice meal is of diagnostic value as an indicator of its antineuritic potency, in which connexion it is interesting to note that Shiuza [1926] has claimed to have produced a beriberi-like disease in rabbits by a diet deficient in K. According to Bliss [1930] foods rich in vitamin B<sub>2</sub>, such as beef, liver, egg-yolk and yeast (also rich sources of aneurin), are all iron-containing foods. Bliss has even gone so far as to suggest that pellagra is an iron deficiency disease since he obtained encouraging results from injection of iron in severe cases.

It would therefore appear likely that some of the symptoms commonly associated with beriberi and usually ascribed to aneurin deficiency may arise from, or be intensified by, deficiencies in certain elements commonly found in foodstuffs in association with aneurin. It is considered that Zn may have some importance in this regard. Curiously enough Hove *et al.* [1937] had found that rats fed on diets containing minimal amounts of Zn developed pellagra-like lesions round the eyes, at the mouth corners and on the feet and lost neck and shoulder hair. These abnormalities did not appear when the animals received a small supplement of Zn.

### SUMMARY

In beriberi the Zn contents of the toe nails, finger nails and skin are reduced to half their normal value and there is a strong indication that the ash content is increased. Data are produced to show that the amount of Zn in natural foodstuffs is positively correlated with that of aneurin. It is suggested that Zn deficiency may be a factor in the beriberi syndrome.

I wish to express my gratitude to Dr L. T. Ride, Professor of Physiology in the University of Hong Kong, who very kindly gave me facilities for working in his laboratories.

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## LII. INHIBITION OF DEHYDROGENASES BY SNAKE VENOM

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*(Received 9 December 1938)*

It has been reported previously [Chain, 1937, 1] that a substance, present in a number of different snake venoms, inhibits in very small concentrations the glycolysis and fermentation of cell-free extracts. The inhibitory effect of this substance is neutralized by the same quantity of antivenin as will neutralize the lethal effects of the venom *in vivo* [Chain, 1937, 2]. The chemical behaviour and mode of action of the inhibitory substance suggested an enzymic action causing the destruction of some integral part of the glycolysing and fermenting complex [Chain & Goldsworthy, 1937].

The following communication deals with the effect of snake venom on oxidation-reduction enzymes. This study was undertaken in the hope of throwing some light on the mechanism of the inhibitory action of the venom on glycolysis and fermentation, since oxidation-reduction enzyme systems play a predominant part in these processes.

### EXPERIMENTAL

As in the previous experiments, dried venom of the Australian black tiger snake (*Notechis scutatus*) was used. The effect of this venom on various dehydrogenases and oxidases was studied.

*Preparation of enzyme solutions.* The lactic, malic,  $\beta$ -hydroxybutyric and succinic dehydrogenases and the cytochrome oxidase were obtained from pig's heart muscle. The muscle was minced twice through a Latapie mincer, washed exhaustively with tap water, ground with sand and *M*/15 phosphate buffer in a mortar and the solution strained through muslin [Ogston & Green, 1935]. The solution was brought to pH 7 and centrifuged for a short time. For some experiments a dried heart muscle preparation was used, prepared by dehydrating the minced washed pig's heart muscle with ice-cold acetone and evaporating off the acetone in a desiccator over  $P_2O_5$ . The dried powder (1.3 g.) was ground with sand and *M*/15 phosphate buffer pH 7.4 (20 ml.) in a mortar, the solution strained through muslin and centrifuged for a short time. The glycerophosphate dehydrogenase was prepared from rabbit's muscle [Green, 1936]. For xanthine oxidase untreated milk was used. The uric acid oxidase was prepared from sheep liver which was minced through the Latapie mincer, dried in a shallow dish *in vacuo* over  $P_2O_5$  and ground in a mortar to a fine powder. The powder (1 g.) was extracted with borate buffer (10 ml.) pH 8.7 for 1 hr. at 37° and the suspension centrifuged. Amino-acid dehydrogenase was prepared from pig's kidney [Krebs, 1935].

*Manometric experiments.* The effect of the venom on the oxidative enzymes was tested in all cases by measuring  $O_2$  uptakes in Warburg vessels at 37° in



O<sub>2</sub>. In addition to 0.3 ml. 40 % KOH for CO<sub>2</sub> absorption in the middle chamber, the vessels contained the following solution:

For lactic acid dehydrogenase:

Side bulb: 0.5 ml. 10 % Na lactate.

Main space: 0.5 ml. 0.1 % cozymase.<sup>1</sup>

0.3 ml. 5 % semicarbazide hydrochloride, neutralized.

0.2 ml. 1 % methylene blue.

1–2 ml. enzyme, according to activity.

For malic and  $\beta$ -hydroxybutyric acid dehydrogenases:

Side bulb: 0.5 ml. substrate (15.6 % Na malate or 6.3 % Na  $\beta$ -hydroxybutyrate)

Main space: 0.5 ml. 0.1 % cozymase.

0.3 ml. *M*/10 Na pyrophosphate.<sup>2</sup>

0.3 ml. *M*/2 hydrazine sulphate, neutralized.

0.3 ml. 1 % methylene blue.

1–2 ml. enzyme, according to activity.

For succinic and  $\alpha$ -glycerophosphate dehydrogenases and cytochrome oxidase:

Side bulb: 0.5 ml. substrate (10 % Na succinate, 4 % ammonium  $\alpha$ -glycerophosphate, 1 % phenylenediamine hydrochloride, neutralized).

Main space: 0.5 ml. 0.1 % cozymase.

0.2 ml. 1 % methylene blue.

0.5–2 ml. enzyme, according to activity.

For xanthine oxidase:

Side bulb: 0.3 ml. 0.1 % xanthine.

Main space: 1.7 ml. milk.

0.5 ml. *M*/5 phosphate buffer pH 7.4.

For amino-acid dehydrogenase:

Side bulb: 0.1 ml. 10 % alanine.

Main space: 1 ml. water.

1 ml. enzyme.

For uric acid oxidase:

Side bulb: 0.3 ml. of a 1 % suspension of uric acid in borate buffer pH 8.7.

Main space: 1 ml. water.

1 ml. enzyme.

Four Warburg vessels were used for each experiment—two control vessels containing the above solutions and two vessels containing snake venom in addition to these solutions. The amount of venom used was usually 1 mg. dissolved in 0.2 ml. water and it was placed together with the other solutions in the main space of the vessels.

<sup>1</sup> I am greatly indebted to Prof. R. A. Peters, Dr S. Ochoa and Dr L. A. Stocken, Department of Biochemistry, Oxford, for gifts of a cozymase preparation of approximately 90 % purity.

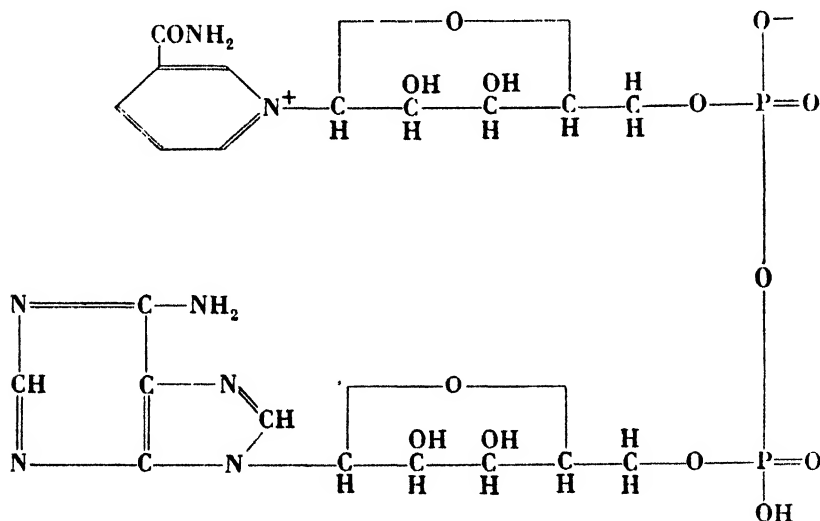
<sup>2</sup> In confirmation of the statement of Green *et al.* [1937] Na pyrophosphate was found to extend considerably the linear period of O<sub>2</sub> uptake with malic and  $\beta$ -hydroxybutyric dehydrogenases.

The results of the tests are summarized in Table I, the figures representing typical values of at least three sets of experiments.

Table I. *Effect of snake venom on oxidative enzymes*

Enzyme	O <sub>2</sub> uptake in 30 min. (μl.)		% inhibition
	Without venom	With venom	
Lactic dehydrogenase	280	0	100
Malic dehydrogenase	180	0	100
β-Hydroxybutyric dehydrogenase	210	0	100
Amino-acid dehydrogenase	142	0	100
Succinic dehydrogenase	600	300	50
α-Glycerophosphate dehydrogenase	85	61	28
Cytochrome oxidase	417	276	34
Xanthine oxidase	167	175	0
Uric acid oxidase	296	308	0

It appears from Table I that lactic, malic, β-hydroxybutyric and amino-acid dehydrogenases are completely inhibited by the venom; the other enzymes are not or only partially inhibited. The four inhibited dehydrogenases have one characteristic in common which differentiates them from the non-inhibited enzymes, namely the need for a coenzyme for their activation. The conclusion therefore naturally suggested itself that the inhibitory effect of the venom might be due to some action on the coenzymes. The need of a coenzyme for the lactic, malic and β-hydroxybutyric dehydrogenases has been known since the systematic study of their properties; it was found to be identical with coenzyme I, which according to Euler & Schlenk [1937] has the structural formula:



The existence of a coenzyme for amino-acid dehydrogenase was discovered only recently by Warburg & Christian [1937] who isolated it in form of its Ba salt and found it to be a flavin-adenine-nucleotide [1938, 1, 2].

By the following experiment it could be shown that coenzyme I is inactivated by black tiger snake venom. Coenzyme I (6 mg.) was dissolved in phosphate buffer pH 7.2 (2 ml.) and venom (2 mg.) in water (0.2 ml.) was added. The mixture was incubated for 1½ hr. at 37°. It was then brought to pH 1.5 with a

few drops of *N* HCl and incubated with cryst. pepsin (1 mg.) for 1 hr. at 37° in order to destroy the venom. The solution was then brought back to pH 7.2 and made up to 3 ml. A solution of coenzyme I (3 mg.) in phosphate buffer (2 ml.), serving as control, was incubated without the addition of venom for 1½ hr. at 37°, then adjusted to pH 1.5, incubated with cryst. pepsin (2 mg.) for 1 hr., brought back to pH 7.2 and made up to 3 ml. The coenzyme I activities of these solutions were tested with the malic acid dehydrogenase system. The arrangement of the test and its results are shown in Table II.

Table II. *Inactivation of coenzyme I by black tiger snake venom*

	Content of vessels in ml.		
	Vessel 1	Vessel 2	Vessel 3
Na malate 15.6%	0.5	0.5	0.5
Coenzyme I, 0.1% incubated as described above	0.2	0.2	—
<i>M</i> /10 Na <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	0.3	0.3	0.3
<i>M</i> /2 Hydrazine sulphate (neutralized)	0.3	0.3	0.3
Methylene blue, 1%	0.2	0.2	0.2
Coenzyme I, incubated with venom as described above	—	1.5	1.5
Water	1.5	—	—
Enzyme	1.0	1.0	1.0
O <sub>2</sub> uptake in 20 min. (μl.)	215	210	7

The O<sub>2</sub> uptake in vessel 3 is very small although it contained 1.5 ml. of the coenzyme I solution which, before incubation with the venom, contained 3.0 mg. Since 0.2 mg. of this cozymase preparation after the treatment described above was sufficient to activate the malic dehydrogenase, as shown by the O<sub>2</sub> uptake in vessel 1, at least 95 % of it must have been inactivated by the venom during the incubation. The O<sub>2</sub> uptake in vessel 2 which is not essentially different from that of vessel 1 shows the effective destruction of the inhibiting substance in the venom by boiling and pepsin treatment.

An indication of the mechanism of the inactivation of coenzyme I by black tiger snake venom is obtained from the fact that CO<sub>2</sub> is evolved when snake venom is allowed to act on coenzyme I in NaHCO<sub>3</sub> solution.

Coenzyme I (2.4 mg.) dissolved in 0.5 % NaHCO<sub>3</sub> (2.2 ml.) was placed in the main space of a Warburg vessel and snake venom (1 mg.) in water (0.2 ml.) and 0.5 % NaHCO<sub>3</sub> (0.1 ml.) in the side bulb. The solutions were saturated with a gas mixture of 95 % N<sub>2</sub> and 5 % CO<sub>2</sub> at 37° and after establishment of equilibrium the venom solution was added to the coenzyme I solution. (CO<sub>2</sub> evolution started immediately and ceased after 45 min. The total amount of CO<sub>2</sub> developed was 141 μl. and corresponds to 1.7 H<sup>+</sup> equivalents per mol. cozymase. The most likely explanation for this is a hydrolytic action of the venom on some of the phosphoric acid linkages in the cozymase molecule with the formation of two new acid groups. Since one of these groups is bound to be weak and therefore will not be dissociated completely at the pH of the solution used in the experiment only 1.7 equivalents of CO<sub>2</sub> are measured instead of two.

The cozymase-inactivating factor in the snake venom which is responsible for the inhibition of glycolysis, fermentation and the four dehydrogenases needing coenzymes is thus shown to belong to the group of phosphoric acid-splitting enzymes, or more specifically, to the class of nucleotidases. It has been known for some time that certain Japanese snake venoms contain a phosphomonoesterase and a phosphodiesterase [Uzawa, 1932; Takahashi, 1932] and recently Gulland & Jackson [1938, 1] in an extensive study have found these enzymes in various proportions in a considerable number of different snake

venoms. The same authors report on the occurrence of an enzyme in several snake venoms which hydrolyses adenosine-5-phosphate and inosine-5-phosphate [1938, 2], and of a nuclease which hydrolyses yeast nucleic acid [1938, 3]. They suggested that some of the toxic effects of the venom might be due to the action of the 5-nucleotidase.

At present it cannot yet be decided by which of the several theoretically possible ways the hydrolysis proceeds. It was found that only a part (approx. 25 %) of the total P is set free in the form of inorganic P during the hydrolysis, and since the cozymase preparation was not 100 % pure it is possible that this inorganic P originates partly from some impurity present in the preparation and not from the cozymase. Obviously the amount of inorganic P liberated is too small to account for the 1.7 H<sup>+</sup> equivalents formed in the hydrolysis. Most probably the first step of the enzymic action is the opening of the pyrophosphate linkage in the coenzyme I molecule with the formation of two mono-ortho-phosphoric esters which then are partly broken down further with the liberation of inorganic P. It is hoped to get more information on this question from a study of the specificity of the phosphatases in the black tiger snake venom.

#### SUMMARY

1. The effect of black tiger snake venom on the following oxidative enzyme systems has been investigated: lactic, malic,  $\beta$ -hydroxybutyric, amino-acid,  $\alpha$ -glycerophosphoric and succinic dehydrogenases: cytochrome, xanthine and uric acid oxidases.

2. The four dehydrogenases which need a coenzyme for their activation, namely the lactic, malic,  $\beta$ -hydroxybutyric and amino-acid dehydrogenases, are completely inhibited by small amounts of black tiger snake venom. The others are not or only partially inhibited.

3. Coenzyme I is shown to be inactivated by black tiger snake venom. The inactivation is shown to be due to enzymic hydrolysis of some of the phosphoric acid linkages in the cozymase molecule with the formation of two new acid groups. The cozymase-inactivating principle in the black tiger snake venom can therefore be classified as a phosphatase or, more specifically, as a nucleotidase.

4. The inactivation of the coenzymes by black tiger snake venom through the action of a nucleotidase is the cause of the inhibitory effect of the venom on glycolysis, on fermentation and on the four dehydrogenases mentioned above.

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# LIII. INVESTIGATIONS ON THE ROOT NODULE BACTERIA OF LEGUMINOUS PLANTS

## XXII. THE EXCRETION PRODUCTS OF ROOT NODULES. THE MECHANISM OF N-FIXATION

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*(Received 25 January 1939)*

THE excretion of N-compounds from leguminous root nodules has been the subject of many years' research in this laboratory. Apart from its great significance in nature and agriculture the excretion has also rendered possible a closer examination of the chemical processes of biological N-fixation which have been completely obscure. As established in several publications<sup>1</sup> from this laboratory the excretion in certain cases assumes such proportions that over 80 % of the total fixed N is excreted from the nodules to the medium by young legumes. This fact and the special composition of the excreted products show that the excreted N-compounds are products of N-fixation and not secondary products of protein decomposition. A thorough chemical investigation of the excretion products was therefore necessary for the elucidation of the mechanism of N-fixation.

The results of our studies hitherto on the chemical composition of excretion products have been described in brief preliminary communications and summarizing lectures. A detailed report of the methods of analysis and of the course of the research has, however, not been presented. In this paper we give such a report.

The biological aspects of the excretion of N-compounds will not be discussed in detail here; we refer in this respect to our earlier contributions.<sup>2</sup> From the general course of the work, however, the following may be mentioned. Peas were grown in a sterile culture system developed by us, using generally N-free quartz sand, and later also cellulose, as a medium. The composition of the nutrient solution was as follows:  $\text{Ca}_3(\text{PO}_4)_2$  0.25 g.,  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  0.25 g.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.39 g.,  $\text{KCl}$  0.25 g.,  $\text{FeCl}_3$  (5 % solution) 3 drops in 1000 ml. of distilled water. Peas were inoculated with an effective strain of legume bacteria isolated from the root nodules of pea (strain HX in this laboratory). The growth of the peas depended, in the absence of nitrogenous fertilizers, entirely on the N-fixation in the root nodules caused by the bacteria. In the sterile system the N-compounds excreted from the root nodules to the medium could decompose only through the action of the legume bacteria, and these decompose the excreted N-compounds only very slowly in a definite manner (cf. below). The

<sup>1</sup> The summary of the results obtained in this laboratory up to the end of 1937 in the field of N-fixation as well as the especially great bearing of legumes on agriculture and human nutrition have been described in A. I. Virtanen's lectures delivered in the University of London and now published in book form under the title, *Cattle Fodder and Human Nutrition with special reference to Biological Nitrogen Fixation*, Cambridge, 1938. References are also included in the book.

<sup>2</sup> Especially we refer to our articles in the *J. agric. Sci.* 27, 332 and 584 (1937).

excretion products could therefore be isolated from the medium at the end of the experiment period unchanged, or changed in a definite manner.

In ordinary pot cultures, even when N-free medium is used for inoculated peas (or other legumes), the excretion products cannot be isolated as such, because various and numerous micro-organisms present in the medium decompose the excretion products thoroughly, forming ammonia and nitrate. The use of a sterile culture system is therefore a necessary condition for the chemical elucidation of excretion products.

The plant experiments have been carried out with Dr Synnöve v. Hausen.

## EXPERIMENTAL

### *Extraction and characterization of the excretion products*

The experiment in 1932 [Virtanen *et al.* 1933] showed that when inoculated peas were grown in quartz sand in sterile culture system, without N-nutrition, and the N-compounds excreted into the medium were isolated by removing the roots carefully from the same, and extracting the sand with boiling water, the extract obtained contained the following N-fractions (Table I).

Table I

	N, mg.	N, % of total N
Total N in the extract	61.50	100.00
Amino-N	47.60	77.40
Ammonia-N	0.00	0.00
Amide-N	2.03	3.30
Volatile bases-N	1.68	2.73
Melanin-N	1.26	2.05

This preliminary experiment already showed that the major part of the nitrogen was amino-N. Since hydrolysis with HCl did not increase the amount of amino-N, the solution contained only free amino-acids and no peptides. Among the individual amino-acids an attempt was made to determine aspartic acid but with negative result. As was noticed later, this failure was due to the fact that the determination was made in the solution after ammonia determination according to Van Slyke, when aspartic acid is precipitated with alcohol and removed from the solution. The presence of volatile bases and melanin-N in the extract led to the assumption that the extraction with boiling water had probably caused decomposition in the primary excretion products.

We therefore made experiments with another excretion method. In a continuously acting apparatus sand was placed in a tinned metal dish, capacity 60 l. A glass tube near the bottom of the dish was led into a boiling flask, in which the water, acidified with  $\text{H}_2\text{SO}_4$  to pH 2-3, was kept boiling. Steam was passed through a condenser, from which the water dropped on to the sand in the metal dish. In the flask the extracted compounds remained in the boiling solution for several days as the extraction in this apparatus was slow. Even this method therefore offered great possibilities for the decomposition of N-compounds. This extraction method was employed in the following experiment.

In the spring of 1935 peas were grown in our greenhouse in 25 Woulff's bottles (3 l.) each containing 4 kg. quartz sand. Two inoculated peas were grown in each bottle. The cultures were harvested in the middle of the flowering period, and the sand was washed from the bottles using as little water as possible. The root particles were carefully removed from the sand. The sand from all bottles (100 kg.) was extracted together for about 100 hr. in the manner

described. The extract was concentrated *in vacuo* to 500 ml. and filtered. The small precipitate contained 10 mg. N, and the clear filtrate, extract II, 577.5 mg. N. N was determined in both cases by the Kjeldahl method.

The filtrate contained the following amounts of amino-N [Van Slyke]:

After 5 min. reaction time:

In 10 ml. (1) 9.55 mg. }  
In 10 ml. (2) 9.49 mg. } In 500 ml. 476.0 mg. amino-N.

After 30 min. reaction time:

In 10 ml. (1) 10.05 mg. }  
In 10 ml. (2) 10.10 mg. } In 500 ml. 504 mg. amino-N.

The increase of amino-N during 30 min. reaction time indicates that the filtrate apparently contained also some other N besides  $\alpha$ -amino-N.

*Volatile bases* were determined in 25 ml. of the solution according to Van Slyke's distillation method. The distillate used 1.1 ml. of 0.1 N  $\text{H}_2\text{SO}_4$  corresponding to 1.54 mg.  $\text{NH}_3\text{-N}$ .

*Ammonia-N* was determined by the same distillation method also in 25 ml. Ammonia was determined in the distillate by Nessler's reagent. 0.3 mg.  $\text{NH}_3\text{-N}$  was found. Accordingly:

500 ml. contained 6.0 mg.  $\text{NH}_3\text{-N}$ ,  
500 ml. contained 24.8 mg. N of other volatile bases.

*Amide-N* was determined in the solution after volatile bases had been distilled according to Van Slyke. Conc. HCl was added to the solution up to 20 %. The mixture was heated on the water bath for 14 hr. after which the major part of the HCl was evaporated *in vacuo*. The residue was nearly neutralized with 20 % NaOH, made alkaline with MgO and  $\text{NH}_3$  was distilled over. The distillate used 0.8 ml. of 0.1 N  $\text{H}_2\text{SO}_4$  corresponding to 1.12 mg.  $\text{NH}_3\text{-N}$ . 500 ml. thus contained 11.2 mg. "amide-N".

Table II illustrates the quantities of different N-fractions in extract II.

Table II

	N, mg.	N, % total N
Total N	577.5	100.0
Amino-N	504.0	87.3
$\text{NH}_3\text{-N}$	6.0	1.0
Volatile bases-N	24.8	4.3
"Amide-N"	11.2	2.0

In this extract the amount of amino-N was considerably higher than in the extract of the preliminary experiment. The extraction method seemed thus to have a marked effect on the composition of the N-compounds. Since in extract II also the N-compounds remained for a long time in boiling solution at pH 2-3, decompositions were still possible.

In extract II the following qualitative tests and determinations were made:

1. Ninhydrin reaction was positive.
2. Folin's amino-acid reaction with 1:2-naphthoquinone-4-sodium sulphonate was positive.
3. Klein's arginine reaction was negative.
4. Winkler's tryptophan reaction was negative.
5. The determination of organic sulphur was negative; thus sulphur-containing amino-acids were not present.
6. Pauly's diazo reaction for histidine and tyrosine was negative.

7. Millon's reaction was negative.

8. The enzyme aspartase (dry preparation of propionic acid bacteria) formed ammonia abundantly in the extract. According to our experiments the said dry preparation does not split off ammonia in the presence of toluene from any amino-acids except from *l*-aspartic acid. Therefore it was evident that *a large quantity of aspartic acid was present* in the extract. Quantitative determination was not made.

9. 100 ml. of the extract (= 115.5 mg. N) neutralized to pH 7 were extracted for 100 hr. with *n*-butyl alcohol in Dakin's vacuum extraction apparatus. Only 0.5 mg. N was removed by the butyl alcohol. Thus it was evident that mono-amino-monocarboxylic acids and proline were not present in the extract.

10. A test was made to find out whether a part of the N in the extract was precipitable with phosphotungstic acid. 50 ml. of the extract (= 57.8 mg. N) were neutralized with NaOH, whereupon 15 g. of phosphotungstic acid were added and the solution brought to the boiling point. The solution was kept for 48 hr. at 37° before the precipitate was separated. Another sample of 50 ml. was taken and 10 ml. of conc. HCl and 15 g. of phosphotungstic acid were added. The solution was kept in the ice-box for 48 hr. N determinations in the precipitates of the two experiments gave the following results:

- (1) 24.0 mg. N or 42 % of total N,
- (2) 28.6 mg. N or 49.7 % of total N.

Since a considerable part of the N was precipitated with phosphotungstic acid, it was evident, in view of the above observations, that the extract contained, in addition to aspartic acid, either diamino-acids or other non- $\alpha$ -amino-acids.

Extract II already contained nearly 90% of the total N in the form of amino-N. The presence of melanin-N and volatile bases indicated that some decomposition had probably taken place during the extraction in boiling solution. Therefore we employed in the following extraction a new method, in which all heating was prevented. This procedure, which we have since used regularly, is illustrated in Fig. 1.

The sand from 10 Woulff's bottles, each containing 4 kg. quartz sand and 2 inoculated peas grown in summer 1935 without N-nutrition, was washed from the bottles with a small amount of water and the roots were removed simultaneously. The sand was extracted in portions of 8 kg. with cold water (about 15–20°) in the apparatus shown in Fig. 1. 3 l. of water acidified with  $H_2SO_4$  to about pH 4 and containing some toluene were mixed well with the sand, whereupon the water was sucked into a filter flask. The extraction was repeated 10 times with the same water. Then a new washing was carried out in the same manner with another 3 l. of water. Each 8 kg. portion of sand was thus finally washed with 6 l. of acid water containing toluene, and the total amount of 40 kg. of sand with 30 l. of water. In this manner 80 % of the excreted N was recovered from the sand (cf. below).

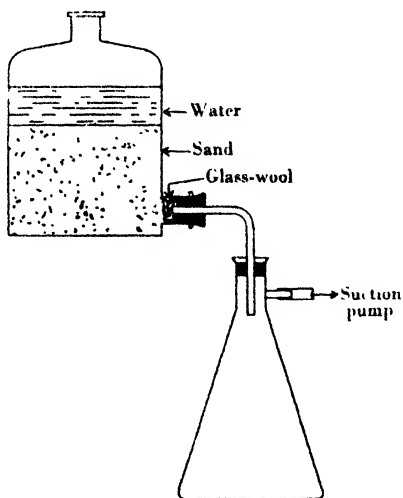


Fig. 1.



The solution was evaporated to 500 ml. *in vacuo* on the water bath at 40°. The small precipitate formed during the evaporation was filtered off, and the filtrate was poured into a 500 ml. measuring glass and made up to this volume with water (extract III). 500 ml. of the extract contained (Table III):

Table III

	N, mg.	N, % of total N
Total N	259.0	100.0
Amino-N (30 min. shaking time)	256.0	98.8
NH <sub>3</sub> -N	Traces	—
Volatile bases-N	Traces	—

The extraction at lower temperature showed that the N excreted from the root nodules was almost entirely amino-N and that the other N-compounds found in the previous extracts were evidently ascribable to the decomposition processes occurring during the extraction.

In this extract the same qualitative determinations were first made as in that described above. The results were identical.

#### *Isolation and characterization of individual excretion products*

*Isolation of aspartic acid.* 5 ml. of 10 % milk of lime and 250 ml. of 96 % alcohol were added, with good stirring, to 20 ml. of extract (= 10.36 mg. N), and kept for 1 hr. (Foreman's precipitate). The mixture was filtered and the precipitate washed carefully with 90 % alcohol, whereupon N-determination was made. The precipitate contained 5.25 mg. N or 50.7 % of the total N. The extract thus contained a monoamino-dicarboxylic acid. Attempts to identify glutamic acid as hydrochloride and as Zn salt, gave negative results. On the other hand we succeeded in isolating aspartic acid as the Cu-salt in the following manner. Amino-acids were precipitated from 50 ml. of extract (= 25.9 mg. N) according to Neuberg. The extract was made alkaline with 10 % Na<sub>2</sub>CO<sub>3</sub>. Small amounts of 25 % Hg acetate and 10 % Na<sub>2</sub>CO<sub>3</sub> were added alternately so that the solution was kept constantly alkaline; 6 vol. of alcohol were added, the solution was filtered and the precipitate suspended in water and decomposed with H<sub>2</sub>S. The filtrate was evaporated *in vacuo* to a small volume (about 25 ml.) and boiled with freshly precipitated Cu(OH)<sub>2</sub>. It was filtered hot and kept in the ice-box. After a while the Cu-salt began to precipitate. Filtration was carried out after some hours, the precipitate was carefully washed with cold water and dried in a desiccator over H<sub>2</sub>SO<sub>4</sub>. The Cu-salt weighed 150 mg. Cu was determined according to Abderhalden & Schnitzler [1927] and N according to micro-Kjeldahl. (Found: Cu, 22.97, 23.30; N, 5.10, 5.03 %. C<sub>4</sub>H<sub>5</sub>O<sub>4</sub>NCu, 4.5 H<sub>2</sub>O requires Cu, 23.06; N, 5.08 %.)

The salt obtained was thus the *Cu salt of aspartic acid*. To confirm the fact, aspartic acid was also determined in the Cu salt by means of the enzyme aspartase.

190 mg. Cu salt were dissolved in 25 ml. of 0.1 N H<sub>2</sub>SO<sub>4</sub> and Cu was precipitated with H<sub>2</sub>S. H<sub>2</sub>S was evaporated from the filtrate. 10 ml. of M/15 phosphate buffer (pH 7), 10 ml. of a suspension of *B. fluorescens liquefaciens* (from 375 mg. of dry bacterial preparation) and 10 ml. of toluene were added. The volume of the solution was made up to 150 ml.

The parallel solution consisted of 100 mg. of *l*-aspartic acid, 10 ml. of phosphate buffer, 10 ml. of bacterial suspension and 10 ml. of toluene, made up to 150 ml.

The control solution for the determination of the NH<sub>3</sub>-content of the bacterial suspension was otherwise the same as the parallel solution, but did not contain aspartic acid.

All three experimental solutions were kept for 5 days at 37°, after which  $\text{NH}_3$  was determined. According to our experience liberation of ammonia from aspartic acid by aspartase reaches practically its maximum during this time, and the reaction  $l$ -aspartic acid  $\rightleftharpoons$  fumaric acid +  $\text{NH}_3$  attains its equilibrium.

The following amounts of ammonia were found in the different solutions:

	Bacterial suspension without aspartic acid	Bacterial suspension + 100 mg. $l$ -aspartic acid (= 10.53 mg. N)	Bacterial suspension + filtrate corre- sponding to 190 mg. Cu salt (= 9.69 mg. N)
$\text{NH}_3$ -N, mg.	4.76	10.50	9.80
$\text{NH}_3$ -N, formed from the substrate	—	5.74	5.04
$\text{NH}_3$ -N, % of the N in the substrate	—	54.50	52.00

The data show convincingly that the isolated Cu salt was Cu  $l$ -aspartate. It is known that aspartase does not split ammonia from  $d$ -aspartic acid.

Several methods were used for the quantitative determination of aspartic acid in the extract. The aspartase method gave the following result.

40 ml. of the extract (= 20.01 mg. N) + 10 ml. of a suspension of *B. fluorescens liquefaciens* (prepared from 375 mg. of dried bacteria) + 10 ml. of  $M/15$  phosphate buffer (pH 7) + 10 ml. of toluene were made up with water to 100 ml. During 5 days at 37°, 5.6 mg.  $\text{NH}_3$ -N were formed (control, with bacterial suspension alone, subtracted). Since, under the same experimental conditions with the same bacterial material,  $l$ -aspartic acid forms 54%  $\text{NH}_3$ -N of the total N, the amount formed in the extract, 5.6 mg.  $\text{NH}_3$ -N, corresponds to 10.4 mg.  $l$ -aspartic acid. Consequently, 52% of the total N in the extract (= 20.01 mg. N) was  $l$ -aspartic acid-N.

When amino-dicarboxylic acids were precipitated from the extract according to Foreman as the Ca salts, 50.7% dicarboxylic acid-N was found. Since the aspartase method gave 52% aspartic acid-N of the total N, it was evident that the whole Foreman-fraction was  $l$ -aspartic acid.

Aspartic acid was determined in still another way, viz. by oxidizing it first to malic acid and further to acetaldehyde. According to Fürth *et al.* [1932], the extract was treated with nitrous acid, followed by permanganate oxidation (the ordinary lactic acid determination). Acetaldehyde is only formed in this manner from  $\alpha$ -alanine and aspartic acid. The acetaldehyde formed corresponded to 48.5% of the total N in the extract. In the absence of  $\alpha$ -alanine this N belongs to  $l$ -aspartic acid.

2 ml. of extract + 75 ml. of water + 0.5 ml. of conc. HCl were heated on the water bath. 15 ml. of 2.5%  $\text{NaNO}_2$  were added drop by drop from the burette during 20 min., after which 15 ml. of 7.5% urea were added in the same manner to remove the excess of nitrite. The solution was made up to 250 ml. and the oxidation with permanganate was accomplished in 50 ml.

1.80 ml. of 0.008  $N$  iodine solution were used, corresponding to 0.317 mg. acetaldehyde. From the whole extract (500 ml. = 259 mg. N) 396.5 mg. acetaldehyde were formed, corresponding to 126 mg. N or 48.5% of the total N.

The different methods, Foreman's precipitation, liberation of ammonia by aspartase and the oxidation of aspartic acid after treatment with nitrous acid, thus gave the following amounts of aspartic acid.

Foreman's precipitation	...	50.7 % aspartic acid-N of the total N
Aspartase method	...	52.0    "    "
Oxidation to malic acid and further to acetaldehyde	...	48.5    "    "

The results of the different methods are in good agreement. Thus approximately 50 % of the N in the whole extract is aspartic acid-N. As was shown by the aspartase method, the aspartic acid in question is the *l*-form. Glutamic acid was not found in the extract.

*Characterization of the amino-acid precipitable with phosphotungstic acid*

Nearly all of the rest of the N in the extract was precipitated with phosphotungstic acid. 50 ml. of the extract (=25.9 mg. N) were precipitated in acid solution with 15 g. of phosphotungstic acid. The solution was heated to boiling and then kept in the ice-box for 48 hr. 12.0 mg. N were precipitated or 47.1 % of the total N. Aspartic acid-N together with the N precipitated with phosphotungstic acid thus formed 97-99 % of the total N. On the basis of the qualitative experiments described above it was evident that the N precipitated with phosphotungstic acid belonged either to a diamino-acid or to a non- $\alpha$ -amino acid. In order to elucidate this point some additional determinations were made.

*Formaldehyde titration.* 8 ml. of the extract, freed from phosphoric acid and CO<sub>2</sub>, used 2 ml. of 0.1 *N* NaOH corresponding to 2.8 mg. amino-N. The whole amount of amino-N was 4.10 mg., thus only 68.3 % of the amino-N had been fixed through formalin. In view of this fact a part of the amino-N must be in a position other than  $\alpha$  with regard to carboxyl.

*Carbon determination* in the extract was made according to Osburn & Werkman [1932]. 25 ml. of extract (=12.95 mg. N) gave 156.0 mg. CO<sub>2</sub>, whence the atomic C : N ratio was 3.8 : 1. In aspartic acid this ratio is 4 to 1, and consequently this ratio in the remaining amino-acid or acids in the extract must be smaller than 3.8 : 1, and in fact considerably smaller because the extract contains also some N-free organic compounds (cf. below).

*Absorption spectrum.* Amino-acids were precipitated according to Neuberg. The mercury precipitate was decomposed with H<sub>2</sub>S, and the solution was evaporated *in vacuo* to a small volume. The final solution contained 10 mg. N in 100 ml. The absorption spectrum in the ultraviolet was photographed. No absorption maxima were noted, showing the absence of tryptophan, tyrosine and phenylalanine. A strong end absorption occurred rising abruptly at 20  $\mu$  but this is characteristic of all aliphatic amino-acids.

*Preparation of a picrate* from the amino-acid fraction precipitable with phosphotungstic acid did not succeed. A dilute alcoholic solution of picric acid formed a precipitate, but it contained mostly potassium picrate, and no pure and characteristic amino-acid picrate could be isolated.

Taking into account all the facts hitherto observed, there was reason to assume that the amino-acid precipitable with phosphotungstic acid was a diamino-acid, probably lysine, provided that the amino-acid in question was a normal amino-acid present in proteins. Attention was therefore paid to the determination of lysine. This amino-acid is difficult to characterize and is therefore often determined indirectly. In order to develop a specific method for the determination of lysine we considered the possibility of splitting the carboxyl group from the lysine with the formation of cadaverine, which is easy to determine. As we have previously reported [1937], an almost quantitative production of CO<sub>2</sub> from lysine was accomplished by a strain of *B. coli* and the corresponding amount of cadaverine was obtained. Applying the same method to our extract we could not obtain cadaverine. From one of our extracts (extract IV), which contained 32.3 mg. N in 30 ml., we precipitated aspartic acid as the Ba salt according to Foreman. The precipitate contained 15.54 mg. N or 48.3 % of total N. Alcohol was evaporated from the filtrate and Ba was removed

as sulphate. The obtained filtrate was evaporated to 25 ml., 2 g. of moist *B. coli* (aseptically isolated) and 25 ml. of glycerol nutrient solution were added. No formation of cadaverine occurred within 3 weeks at 37°. From the parallel experiment, to which lysine was added, 90% of the theoretical amount of cadaverine was isolated. This proved that the N-fraction precipitating with phosphotungstic acid could not contain lysine. Putrescine, which is formed from ornithine by the same bacteria, was also excluded.

In the absence of all the known  $\alpha$ -amino-acids, which might possibly have passed into the N-fraction precipitable with phosphotungstic acid, the unknown amino-acid must be one which is not found among the products of protein hydrolysis. Our attention was now directed to  $\beta$ -alanine. It appeared that the *legume bacteria split off quantitatively one carboryl group from l-aspartic acid with the production of  $\beta$ -alanine* [Virtanen & Laine, 1937]. The presence of  $\beta$ -alanine among the excretion products of root nodules was thus quite natural. The following methods were used for the identification of  $\beta$ -alanine in our extracts.

*The formation of ethyl acrylate.* All the extracts investigated, and their N-fractions precipitable with phosphotungstic acid, gave, when treated with HCl and alcohol according to Abderhalden & Fodor [1913], a strong smell of ethyl acrylate.  $\beta$ -Alanine can be qualitatively determined in this manner.

*The butyric acid compound of  $\beta$ -alanine.* Przylecki & Kasprzyk [1937] have introduced a method for the characterization of basic amino-acids by means of their butyric acid compounds. 100 ml. of the extract (pH about 4) from sterile sand cultures of inoculated peas, containing 69.0 mg. N, were extracted in a percolator with ether for 3 days to remove the ether-soluble substances (cf. below). The aqueous solution, containing all the amino-N, was evaporated *in vacuo* almost to dryness, whereupon anhydrous  $\text{Na}_2\text{CO}_3$  and anhydrous  $\text{Na}_2\text{SO}_4$  were added. The dry mass was extracted in a Soxhlet apparatus with abs. methyl alcohol for 48 hr. 28.1 mg. N or 40.7% of the total N in the extract were obtained in the methyl alcohol, which was evaporated to dryness *in vacuo*. The residue was extracted with abs. butyric acid for 4 hr. and then filtered. The butyric acid solution contained 20 mg. N. Ether was added to the solution, causing the formation of a fluffy precipitate containing N. On washing with ether the precipitate became syrupy. It was dissolved again in butyric acid and precipitated once more with ether. The precipitate was washed with ether and dried in a desiccator. Its m.p. was about 180°. The butyric acid compound prepared from  $\beta$ -alanine melted at 204°. The mixture of both melted at 190°. On microscopic examination the butyric acid compound prepared from the extract was found to contain crystals typical of the butyric acid ester of  $\beta$ -alanine. A part of the preparation was, however, amorphous and the low m.p. was evidently due to this. Various salts in the extract originating from the nutrient solution of peas, were partly dissolved in methyl alcohol and butyric acid, and obviously prevented complete purification of the butyric acid compound.

The butyric acid compound gave, when treated with HCl and alcohol, a distinct smell of ethyl acrylate.

*Oxidation of the extract with ninhydrin.*  $\alpha$ -Alanine is quantitatively oxidized with ninhydrin to acetaldehyde; aspartic acid forms at the most 10% of the theoretical amount; other normal amino-acids do not form acetaldehyde, whereas  $\beta$ -alanine forms 10–34% of the theoretical amount in different experiments [Virtanen & Laine 1938].

From an extract of quartz sand cultures of peas (extract V) aspartic acid was removed according to Foreman. The filtrate which contained 12.1 mg. N gave, on oxidation with ninhydrin, 6.46 mg. acetaldehyde or 17% of the theoretical

amount (1 N atom corresponds to 1 mol. acetaldehyde). This result therefore supports the conclusion that  $\beta$ -alanine forms at least the major part of the N-fraction precipitable with phosphotungstic acid.

Whether the whole fraction consists of  $\beta$ -alanine does not directly appear from our experiments. The absence of all known amino-acids implies, however, that  $\beta$ -alanine is the only amino-acid in the fraction. This fact is confirmed also by the ratio of aspartic acid to the fraction precipitable with phosphotungstic acid in the sand during different stages of growth of the pea (cf. below).

#### *Nitrite- and oxime-N in the extract*

As has been mentioned above, the extraction of quartz sand cultures of inoculated peas with acid water at room temperature produces extracts in which nearly all (90–99%) the N is amino-N as determined according to Van Slyke using 30 min. reaction time. In the autumn of 1935 we detected the presence of  $\text{NO}_2\text{-N}$  in the extract by means of  $\alpha$ -naphthylamine and sulphanilic acid according to Blom [1926]—later we used the more sensitive reagent atoxycocaine instead of sulphanilic acid according to Jendrassik & Falcik-Szabó [1933]. Before extraction also some nitrite was generally found in the sand cultures. In every case, however, the  $\text{NO}_2$ -content of the concentrated extract was many times higher than before extraction of the sand. During extraction and especially during concentration of the extract some N-compounds must thus have formed  $\text{NO}_2\text{-N}$ . This is evident from the following experiment.

Sterile system. 2 Woulff's bottles; 4 kg. quartz sand; 2 inoculated peas in each bottle. N-free nutrient solution. After 40 days' growth the plants were harvested and roots carefully removed from sand.

4 peas		
Dry wt., g.	N, mg.	N, excreted in sand, mg.
2.040	60.6	90.9

In this experiment much more N was excreted into the sand than had been taken up by peas. Solution sample taken from the sand gave a very slight nitrite reaction.

The sand was washed five times, in the apparatus described above, with a total of 16 l. tap water. After every washing  $\text{NO}_2\text{-N}$  was determined in the washings, which were then evaporated *in vacuo* to 100 ml. The air passed through the solution during evaporation was washed with alkali to remove possible N-oxides. The washing waters contained the following amounts of  $\text{NO}_2\text{-N}$ :

1.	4 l. washing water	0.16 mg. $\text{NO}_2\text{-N}$
2.	3       "	0.15       "
3.	3       "	0.05       "
4.	3       "	0.01       "
5.	3       "	0.00       "
Total		0.37 mg. $\text{NO}_2\text{-N}$

100 ml. of concentrated extract contained 1.72 mg.  $\text{NO}_2\text{-N}$ ; thus its amount had increased during concentration from 0.37 to 1.72 mg. Also during the washing of the sand a considerable increase of  $\text{NO}_2\text{-N}$  had already occurred, but the amount cannot be expressed quantitatively, since the determination of nitrite in sand was not quantitative.

Examining the course of formation of  $\text{NO}_2\text{-N}$  we noticed that Endres [1935] had recently found oxime-N in *Azotobacter* cultures. Employing this method (hydrolysis of the oxime at  $100^\circ$  for 6 hr. in 3 N  $\text{H}_2\text{SO}_4$ , followed by oxidation of the hydroxylamine formed with iodine to nitrite at room temperature) we found oxime-N in our extract also. In sand cultures it usually amounted to 1–2% of

the total excreted N.  $\text{NO}_2\text{-N}$  was apparently formed from oxime-N, since in the concentrated extract oxime-N was decreased and  $\text{NO}_2\text{-N}$  increased. Later, when the chemical nature of the oxime was known, it was possible to prove experimentally the formation of  $\text{NO}_2\text{-N}$  in sterile cultures of peas from the oxime in question (cf. below).

Since the oxime is partly decomposed during slow extraction and particularly during long vacuum evaporations of large volumes of liquid, we started to use finely divided cellulose as a medium for the peas in order to isolate the oxime. 500 g. finely divided cellulose were placed, together with a sufficient amount of N-free nutrient solution, in a 1.5 l. suction flask. Two peas were grown in each flask. The sterile system was the same as with sand. All the excreted N-compounds were removed from the cellulose by 2-3 washings with a little water. In our experiments the nutrient solution was first pressed from the cellulose, after which the latter was washed three times with 1.5 l. of distilled water, altogether 4.5 l. The washings were added to the nutrient solution and evaporated as rapidly as possible *in vacuo* to 100 ml. Even then decomposition of oxime occurred to some extent as indicated by the following experiment.

Two parallel experiments with inoculated peas. Period of growth, 1 Mar.-23 April, 1936. Cellulose used as a medium.

No. of exp.	Peas		Excreted N, mg.	Medium		
	Dry wt., g.	N, mg.*		Oxime-N, mg.	$\text{NO}_2\text{-N}$ , mg.	Oxime-N + $\text{NO}_2\text{-N}$ % of excreted N
1	1.370	36.5	7.3	0.06	0.10	2.2
2	1.291	34.4	6.7	0.12	Traces	1.8

\* N in seeds is subtracted.

The total amount of oxime-N and nitrite-N in numerous cellulose cultures investigated was regularly 1-2 % of the excreted N. Only in one experiment did this amount rise to 10 %. In cultures of ripening peas, when N-fixation in the root nodules ceases, oxime-N is no longer found. Amino-N amounted, in cellulose cultures, to 95-98 % of the excreted N.

The isolation and characterization of the oxime was accomplished as follows.

#### *Isolation and characterization of oxime*

*Isolation as Cu salt.* From several cellulose cultures of peas the N excreted from the root nodules was extracted with water in the manner outlined above. 200 ml. of concentrated extract were made acid and extracted for 24 hr. in a percolator with ether, which removed 4 mg. oxime-N. The ether was evaporated off and the residue dissolved in a little water. A small amount of this solution was precipitated according to Foreman; all the oxime-N was thereby removed, whence the oxime was apparently also a dicarboxylic acid.

From the major part of the solution the Cu salt was prepared, by addition of 10 %  $\text{CuSO}_4$  and alcohol. The precipitated Cu salt was dried at 50°. 20 mg. of green-coloured salt were obtained.

For comparison the Cu salt was prepared in the same way from synthetic oximinosuccinic acid. Both salts were similar in appearance.

*Analyses:* Cu was determined according to Abderhalden & Schnitzler [1927].

Cu salt prepared from the extract gave on analysis 31.6 % Cu.

Cu salt prepared from oximinosuccinic acid gave on analysis 31.1 % Cu.

The analytical data are in very good agreement. The Cu salt of the oximinosuccinic acid contains theoretically 30.5 % Cu. Low Kjeldahl N-values were

found for both the synthetic Cu salt and that of oximinosuccinic acid isolated from the extract owing to the fact that only a small part of the oxime-N is determined by this method. Both the Cu salts contained oxime-N as determined according to Endres [1935], but the values were in both cases too low owing to the fact that oxime-N is partly oxidized in the presence of Cu. In the synthetic Cu salt 66 % and in the isolated Cu salt 79.6 % of the theoretical amount of oxime-N were found.

*Characterization of oxime through its reduction to aspartic acid.* Ether solution containing 2 mg. oxime-N, prepared from pea cultures as described above, was evaporated and the residue dissolved in 10 ml. of methyl alcohol. A small amount of finely divided platinum was prepared, and saturated with hydrogen in the cold hydrogenation apparatus of Hückel. The methyl alcoholic solution of the oxime was added and hydrogenated. The hydrogen uptake was 60 ml. in 3 hr. The solution was filtered and evaporated to dryness, the residue dissolved in water and extracted thoroughly with ether in a percolator to remove N-free substances such as succinic acid formed from fumaric acid (cf. below). The aqueous solution was evaporated to a small volume and boiled with freshly precipitated  $\text{Cu}(\text{OH})_2$ . It was filtered hot and kept in the ice-box. A green-coloured Cu salt separated which, after drying in a desiccator, weighed 10 mg. and gave on analysis 23.20 % Cu and 5.66 % N. Cu-aspartate requires 23.06 % Cu and 5.08 % N. Since aspartic acid can be formed on reduction only from oximinosuccinic acid, this must have been the oxime isolated with ether from the cellulose culture of peas.

#### *N-free C-compounds in the extract*

*N-free dicarboxylic acids.* After reduction of the oxime the N-free residue left in the ether was dissolved in water and precipitated with  $\text{AgNO}_3$ , yielding the *Ag salt of succinic acid*. The great excess of hydrogen taken up in the reduction had therefore been used in the reduction of fumaric acid to succinic acid.

Fumaric acid could be isolated with ether from an extract of sand culture of peas, which was kept until all the oxime was decomposed. The amount of fumaric acid was small, 25 mg. of the crude substance being obtained from an extract which contained 120 mg. N. It is possible that in addition to fumaric acid some malic and succinic acids were present in the extract, but these were not investigated more closely.

#### *Amount of aspartic acid among the excretion products at different stages of growth*

When sand was washed with water to isolate the excreted N-compounds, it was found that these were very difficult to remove. The thorough extraction, outlined above, with cold water slightly acidified with  $\text{H}_2\text{SO}_4$  (pH 3-4) usually led to the recovery of about 80 % of the excreted N-compounds in the solution. The possibility therefore existed that the remaining 20 % of the N was to be found in compounds other than those in the extract. This fact deserved full attention when theoretical conclusions were drawn.

We therefore tried to transfer all the N-compounds from the medium to the aqueous solution. This was easily attained when finely divided cellulose was used as a medium. Already with two washings all excreted N was recovered from the cellulose in the aqueous solution. The excretion was, however, lower in cellulose cultures than in sand cultures, varying usually from 10 to 20 % of the total fixed N, so that in order to obtain the same quantity of N for investigation much more cellulose cultures would have been required than sand cultures.

Besides, since quartz sand was mostly used as a medium in our experiments, we tried to study as completely as possible the particular N-compounds excreted in such media.

In continuing the extraction experiments we could ascertain that the extraction was generally the more complete the earlier the peas were harvested. Using for extraction water which was acidified with HCl to pH 3-4 we succeeded in recovering from young pea cultures, harvested before flowering, practically all the excreted N in the aqueous solution, as shown by the following experiment (Table IV).

Table IV

3 l. Woulff's bottles; 4.8 kg. dry quartz sand and 2 l. N-free nutrient solution in each bottle. 2 Torsdag peas in each bottle, inoculated with strain HX. Period of growth 17 Sept.-22 Oct. 1937. Plants were still not in flower at the end of the experiment. Roots were removed and the sand from all bottles was mixed together. 8 kg. of sand were extracted at a time with cold water in the apparatus shown in Fig. 1. Water was acidified with HCl to pH 3-4. All aqueous extracts were evaporated together *in vacuo* to 200 ml.

No. of exp.	Dry wt. of plants g.	N in plants mg.*	N excreted in sand, mg.*	Total fixed N, mg.	Extent of excretion	In the aqueous extract		
						N, mg.	N, % of the excreted N in sand	Aspartic acid-N % of total excreted N
1	1.116	14.5	16.3	30.8	52.9	64.6	99.8	74.7
2	0.940	14.1	5.0	15.0	33.3			
3	1.115	11.5	16.3	27.8	58.6			
4	0.936	10.4	10.1	20.5	49.3			
5	1.115	14.9	5.0	20.0	25.0			
6	1.382	18.2	5.0	23.2	21.5			
7	0.699	13.8	7.0	10.8	64.8			

\* Control subtracted.

In this experiment 74.7% of excreted N was *l*-aspartic acid-N. This amount was appreciably higher than in previous experiments. The difference is due, as will be seen from the data below, to the fact that aspartic acid decreases while  $\beta$ -alanine increases at later stages of growth, a result which is to be expected. Since in the earlier experiments the plants had been in flower, the amount of aspartic acid was already smaller.

In the extract obtained from the experiments presented in Table IV the same qualitative amino-acid analysis was carried out as described earlier in connexion with extract II; the results were identical. With the exception of aspartic acid the extract thus contained no other amino-acids normally present in proteins.  $\beta$ -Alanine could be detected in the extract, since acetaldehyde was formed in the ninhydrin reaction after removal of aspartic acid. In addition, the extract contained some nitrite- and oxime-N:

	% of total N
N precipitated according to Foreman ( <i>l</i> -aspartic acid-N)	74.7
N precipitated with phosphotungstic acid ( $\beta$ -alanine, calculated)	23
Oxime- and nitrite-N	2

On the basis of this result, the root nodules would not excrete other N-compounds than those found in our investigations, unless the phosphotungstic acid fraction contains some unknown amino-acid in addition to  $\beta$ -alanine. The large amount of aspartic acid in the extract is obviously due to the fact that the cultures were harvested at a very early stage, before flowering. The following series of experiments (Table V), in which the cultures were harvested at different



Table V

The experiment was arranged in the same manner as that in Table IV.

Period of growth	Stage of growth	Dry wt. of plants, g.	N in plants, mg.*	N excreted in sand mg.*	Total fixed N, mg.	Extent of excretion	In the aqueous extract		
							N, mg.	N, % of the excreted N in sand	Aspartic acid-N, % of the N in the extract
30. iii.- 24. iv. 37	Before flowering	1.319	28.0	9.1	37.1	24.5	28.8	95.1	63.2
		1.648	44.3	13.0	56.3	23.1			
		1.449	33.3	8.2	41.5	19.7			
30. iii.- 3. v. 37	At start of flowering	3.456	105.4	35.4	140.8	25.2	118.3	90.9	51.7
		2.947	79.9	51.8	131.7	39.3			
		4.011	118.6	43.7	162.3	27.0			
30. iii.- 13. v. 37	In full bloom	3.438	112.1	16.3	128.4	12.8	56.1	87.2	47.0
		3.449	104.0	20.6	124.6	16.5			
		4.460	122.9	27.4	150.3	18.2			
30. iii.- 28. v. 37	Pods developed	7.904	167.2	7.7	174.9	4.4	33.1	85.0	35.7
		8.453	159.9	20.2	180.1	11.2			
		8.382	204.4	11.0	215.8	5.1			

\* N of the uninoculated controls (14.0 mg.) subtracted.

times, shows clearly that the percentage of aspartic acid-N in the excreted N decreases while  $\beta$ -alanine increases towards the end of the growth, as is to be expected.

This experiment illustrates clearly that the aspartic acid content is higher in younger cultures than in older ones. The experiment also shows that during later stages of growth the pea is able to absorb from the medium through its roots the N-compounds which have been excreted at the earlier stages. In this connexion aspartic acid is of major importance; according to our findings it is an excellent N-source for the legumes.

It is interesting that in older cultures of inoculated peas oxime-N is no longer found.

In 1935-36 we determined the excretion products regularly in pea cultures harvested at flowering stage and obtained extracts which contained about 50% aspartic acid-N. The above data show, however, that early in growth *l*-aspartic acid is the main excretion product, and that it is later partly replaced by  $\beta$ -alanine, so that the amount of *l*-aspartic acid decreases continuously with the age of the cultures. In associated cultures of inoculated peas and non-legumes e.g. barley (sterile system) the amount of aspartic acid is generally higher than in cultures of peas alone, since the non-legume cannot utilize aspartic acid appreciably but can use  $\beta$ -alanine.

## DISCUSSION

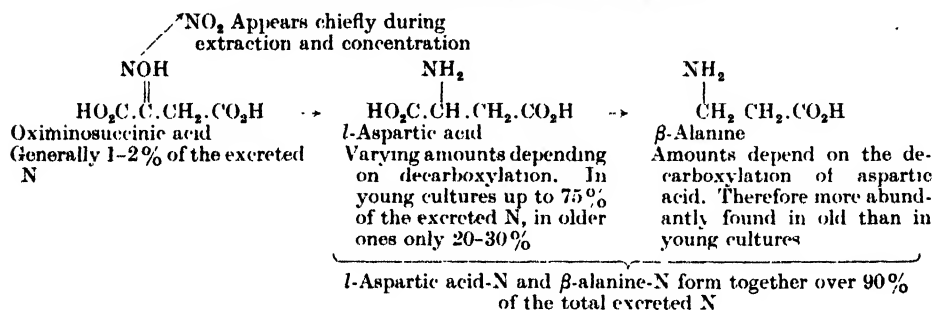
In the medium of inoculated peas grown in a sterile culture system N-compounds appear immediately after nodule formation. The N-compounds have been shown to be excreted from the root nodules and not from the roots. Their amount may increase so much that the major part of the total fixed N is diffused from the root nodules to the medium and the host plant can utilize only a small part of the fixed N. Such being the case, the chemical nature of the excreted N-compounds is of especial interest from the standpoint of the mechanism of N-fixation.

The excreted N is chiefly amino-N. In young pea cultures the major part of the excreted N is accounted for as *l*-aspartic acid. In addition to aspartic acid

also an amino-acid precipitable by phosphotungstic acid has been found among the excreted N-compounds and has been shown to be  $\beta$ -alanine, which is formed from *l*-aspartic acid by the legume bacteria. Other amino-acids have not been found in the medium of inoculated peas. In addition to amino-N the medium contains some  $>C:NOH-N$  and in most cases also some  $NO_2-N$ ; their total amount together is generally 1–2% of the total excreted N. The nitrite-N is formed from oxime-N, the oxime in question being oximinosuccinic acid.

The excreted N-compounds and their mutual relations appear from the following:

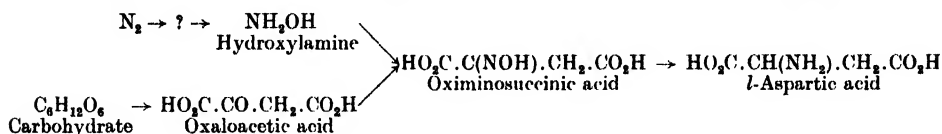
*The excretion products of the root nodules*



Taking into account the often exceptionally high rate of excretion as well as the chemical composition of the excreted N-compounds and their mutual relations, the excreted compounds must be regarded as the products of N-fixation. As  $\beta$ -alanine is a secondary decomposition product of aspartic acid, the actual percentage of aspartic acid-N in excreted N is over 90%. On the other hand, as the ratio of excreted N to total fixed N may rise to 80%, it can be said that approximately 75% of the total fixed N may be excreted in the form of *l*-aspartic acid. Thus it is impossible that the excretion products of the root nodules are products of decomposition of bacterial proteins, since the content of aspartic acid in proteins is far from such amounts, being generally below 10%. According to our determination the N in root nodules contains about 10% aspartic acid-N. In addition the legumes use aspartic acid excellently as their N-source and on the basis of the facts hitherto known it seems likely that the legumes receive their N-nutrition from the root nodules actually in the form of aspartic acid. Thus the percentage of aspartic acid-N in the total fixed N would rise close to 100%. *The fixation of atmospheric N in the root nodules thus leads to l-aspartic acid, which consequently is a primary fundamental amino-acid.*

The mechanism of the formation of aspartic acid is explained by the isolation of oximinosuccinic acid. This oxime, which is easily reduced to aspartic acid, is obviously the precursor of aspartic acid. Oximinosuccinic acid is formed from hydroxylamine and oxaloacetic acid. Even in extremely dilute solutions oxaloacetic acid and hydroxylamine react instantaneously at slightly acid or neutral reaction so that hydroxylamine cannot be detected in a solution which contains an excess of oxaloacetic acid. No other carbonyl compounds investigated by us react so vigorously with hydroxylamine as does oxaloacetic acid. Oximinosuccinic acid must therefore be formed in the root nodules from hydroxylamine and oxaloacetic acid. Experimentally we have been able to show that oxaloacetic acid is found in leguminous plants; the host plant thus supplies the acceptor for hydroxylamine. The fact that hydroxylamine has not been found among the excretion products of root nodules is easily explicable, owing to its rapid

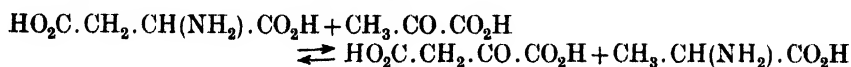
reaction with oxaloacetic acid. On the basis of the experimental facts the course of biological N-fixation occurring in the leguminous root nodules is as follows:



This course of the reaction is in agreement with all the facts hitherto known; the results cannot be interpreted otherwise on the basis of the present knowledge. How hydroxylamine is formed from atmospheric nitrogen has not yet been experimentally proved. It can be assumed hypothetically that a di-imide is formed as a primary reduction product ( $\text{N}_2 \rightarrow \text{HN}=\text{NH}$ ) which then forms hydroxylamine by addition of two water molecules. The reduction of the nitrogen molecule, "N-fixation" in a stricter sense, requires obviously a specific enzyme, which is found in the N-fixing bacteria. The reaction of hydroxylamine with oxaloacetic acid is a non-enzymic process. On the other hand, the reduction of oximosuccinic acid to l-aspartic acid is an enzymic reaction. It has not yet been discovered from which compounds in the plant the hydrogen required for the reduction is transferred to the substrate to be reduced, and which enzymes act in the reduction process.

On the basis of the above facts N-fixation requires the presence of oxaloacetic acid. The important role of this  $\text{C}_4$ -dicarboxylic acid in N-fixation offers an explanation of the symbiosis of legumes and intranodular bacteria, which has hitherto remained mysterious. It has been possible to show synthetically the decisive role of oxaloacetic acid in N-fixation and thus to throw more light on our idea of the reaction mechanism of N-fixation. Excised root nodules do not fix nitrogen in aqueous solution and even in solutions containing glucose N-fixation is either nil or very slight, but in a neutral solution of oxaloacetic acid a vigorous N-fixation occurs already in a few hours [Virtanen & Laine 1937]; simultaneously oxime-N and amino-N appear in the solution. In a series of 25 experiments distinct N-fixation has been accomplished in all the experiments with excised root nodules in presence of oxaloacetic acid. We are going to describe these experiments more closely in another communication.

Aspartic acid is, according to our results, a primary amino-acid formed in the N-fixation. Other amino-acids must therefore be formed from this fundamental amino-acid. In the pea plant the reaction:



takes place rapidly, so that the formation of other amino-acids from aspartic acid is easily understood [Virtanen & Laine, 1938, 2]. In plants the reamination (*Umaminierung*) occurs at least partly in the same manner as, according to Braunstein & Kritzman [1937], it does in animal tissues. It is surprising that no glutamic acid is formed in N-fixation by root nodules, although glutamic acid occupies a special position in amino-acid synthesis. In v. Euler's laboratory [Adler *et al.* 1938] several important investigations have been carried out during the last year on the dehydrogenation of glutamic acid and on the reamination taking place thereby, which according to these findings is caused in micro-organisms and plants by a specific glutamic acid dehydrogenase.

The reaction mechanism of the N-fixation occurring in free-living *Azotobacter* is obviously similar to that occurring in the intranodular legume bacteria. The

excretion of N-compounds by *Azotobacter* is, however, so slight that it would be impossible to conclude anything from it, if the results attained with legume bacteria did not justify the conclusions by analogy. The great difference in the excretion by *Azotobacter* and by intranodular legume bacteria is obviously due to the fact that the former uses the products of N-fixation largely for the formation of its cell protein, while the latter excrete the major part of them. We shall give later a detailed report of our experiments with *Azotobacter*.

#### SUMMARY

The N-compounds excreted from the root nodules of leguminous plants have been isolated and characterized.

Over 90% of the excreted N is amino-N. In addition, 1-2% oxime-N and some nitrite-N is found.

The major part of the amino-N is present as *l*-aspartic acid, if peas are harvested at a young stage, long before flowering. In ageing cultures the amount of aspartic acid decreases.

The other amino-acid found among the excretion products is  $\beta$ -alanine which is slowly formed from *l*-aspartic acid by the legume bacteria. Its formation explains the decrease of aspartic acid in ageing cultures.

The oxime appearing among the excretion products is *oximinosuccinic acid*; nitrite-N is formed from this oxime.

Some *fumaric acid* is detected as an N-free excretion product.

The often exceptionally high amount of excreted N (60-80% of the total fixed N) and the special nature of the excreted N-compounds prove that these N-compounds are products of N-fixation. An idea has thus been obtained, on the basis of the excreted N-compounds, of the mechanism of biological N-fixation.

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# LIV. THE ULTRAVIOLET ABSORPTION OF SHEEP THYROGLOBULIN

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ANALYSIS of the ultraviolet absorption curve of thyroglobulin in terms of the absorption of its iodine-containing components thyroxine and diiodotyrosine leads to interesting results. The absorption spectra of these amino-acids have previously been measured by Hicks [1925], Abderhalden [1927] and Heidt [1936]. In Fig. 1 our own results are given. The measurements were made on alkaline solutions (0.02 *N* NaOH) and acid solutions (diiodotyrosine in 0.1 *N* HCl; thyroxine in 0.014 *N* HCl). At the pH of the alkaline solutions ( $pH \geq pK + 2$ ) the phenolic hydroxyl, the ionization of which governs the change of absorption spectrum from the acid form to the alkaline form [Stenström & Reinhardt, 1925, 2], is almost completely ionized whereas in the acid solutions ( $pH \leq pK - 2$ ) it is almost completely unionized. For diiodotyrosine

$$pK = 6.2.$$

In these absorption curves no "fine structure" (vibrational structure) is present. We cannot, therefore, expect to be able to detect the presence of thyroxine and diiodotyrosine in thyroglobulin by characteristic peaks in the thyroglobulin curve. This conclusion is confirmed by the smoothness of the absorption curves of thyroglobulin at  $pH$  8.0 and  $pH$  1.3 (Fig. 2). The curves show a single maximum at 280  $m\mu$ —the well-known protein band due to tyrosine and tryptophan. No maximum can be distinguished in the region of the alkaline absorption bands of thyroxine and diiodotyrosine from 350 to 300  $m\mu$ .

Two methods of quantitative analysis are possible. First, one can compare the thyroglobulin absorption curve at  $pH$  8.0 with that of serum globulin at  $pH$  8.0 (curve 2, Fig. 2). This method is open to objections, e.g. the difference between serum globulin and the globulin in thyroglobulin. A second method has proved to be more satisfactory. Here the different compounds are not characterized in the usual way by an absorption curve which represents the (molecular) extinction coefficient as a function of the wave-length, but by a curve which represents the so-called differential (molecular) extinction coefficient as a function of the wave-length.

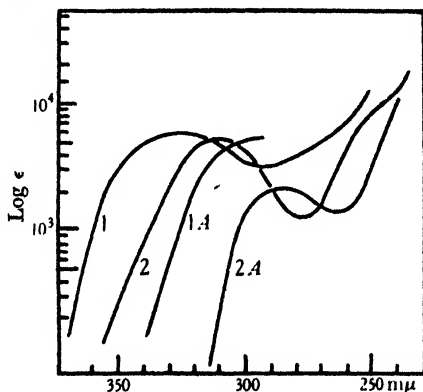


Fig. 1. Molecular extinction coefficients of: 1, thyroxine in 0.02 *N* NaOH; 1A, thyroxine in 0.014 *N* HCl; 2, diiodotyrosine in 0.02 *N* NaOH; 2A, diiodotyrosine in 0.1 *N* HCl.

The differential extinction coefficient  $\delta$  at a given wave-length is defined as the difference between the alkaline extinction coefficient  $K$  (at  $pH \geq pK + 2$ ) and the acid extinction coefficient  $K'$  (at  $pH \leq pK - 2$ ). In a similar way the differential molecular extinction coefficient  $\delta_\epsilon$  is defined as the difference between the alkaline molecular extinction coefficient  $\epsilon$  and the acid molecular extinction coefficient  $\epsilon'$ .

The advantage of the use of  $\delta$  instead of  $K$  or  $K'$  lies in the fact that  $\delta$  is zero for all compounds which show no shift in the absorption spectrum in the given pH range.

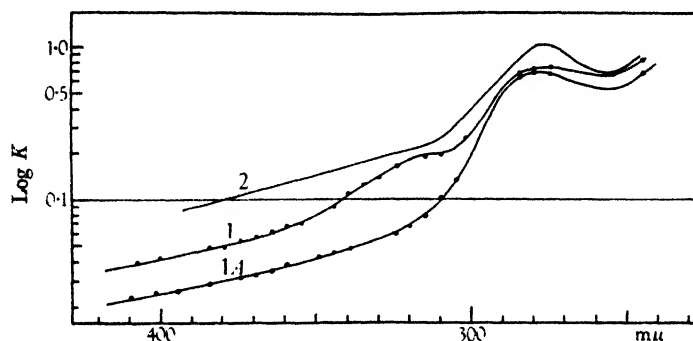


Fig. 2. 0.7% thyroglobulin solution at pH 8.0 (1) and at pH 1.3 (1.4). 1.0% sheep serum globulin (2).

By means of the  $\delta$  curves it has been possible to analyse the thyroglobulin curves in the region 400–300 mμ. An extension of the analysis to wave-lengths below 300 mμ failed because of masking by the protein bands. In Fig. 3 are

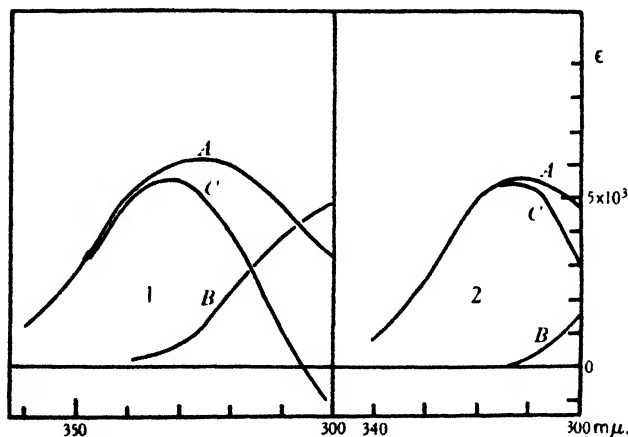


Fig. 3. 1, Molecular extinction of thyroxine; 2, molecular extinction of diiodotyrosine; A, in alkali; B, in acid; C, molecular differential extinction.

represented the  $\delta_\epsilon$ ,  $\epsilon$  and  $\epsilon'$  curves of thyroxine (1) and diiodotyrosine (2). The values of the molecular (differential) extinction coefficients are given in Table I.

As may be noticed, the  $\delta_\epsilon$  bands are more sharply defined than the  $\epsilon$  bands and for this reason they are more apt to characterize the compounds. The following is a description of the way in which the  $\delta$  curve of the iodine compounds

Table I

Thyroxine							
$\lambda$ (m $\mu$ )	360	350	340	330	320	310	300
$10^{-3} \epsilon$	1.1	2.6	5.0	6.1	6.1	4.9	3.4
$10^{-3} \epsilon'$	—	—	0.1	0.4	2.1	3.9	5.0
$10^{-3} \delta_s$	1.1	2.6	4.9	5.7	4.0	1.0	-1.6
Diiodotyrosine							
$10^{-3} \epsilon$	—	—	0.8	2.4	4.9	5.6	4.5
$10^{-3} \epsilon'$	—	—	—	—	—	0.3	1.5
$10^{-3} \delta_s$	—	—	0.8	2.4	4.9	5.3	3.0

in thyroglobulin can be derived from the absorption curves of thyroglobulin at pH 8 and pH 1.3.

In the region from 400 to 300 m $\mu$  the absorption  $K_t$  of thyroglobulin is determined by the following factors (Fig. 2, curves I and I.A):

(a) Scattering of light:  $K_s$  (present from 400 to 300 m $\mu$ ).

(b) Absorption of the iodine components  $K_i$  (present from 370 to 300 m $\mu$ ).

(c) Absorption of the globulin  $K_g$  (present from 320 to 300 m $\mu$ ).

Each of these quantities will be discussed briefly.

$K_s$  is caused by the presence of larger protein particles in the solution which cannot be centrifuged down even at 15,000 rev./min. The properties of this scattering have been studied in solutions of serum globulin (Fig. 2, curve 2); there is no reason why the scattering of serum globulin should differ much from the scattering of thyroglobulin. The serum globulin used was prepared in a way similar to that used for the preparation of thyroglobulin.

The scattering was found to be proportional to  $1/\lambda^4$ . Thus the scattering could be described by the Rayleigh-Jeans formula for the scattering of particles which are small compared with the wave-length of the incident light.

$K_i$  occurs below 380 m $\mu$  in the thyroglobulin curve. Above this wave-length the thyroglobulin curve is similar to the serum globulin absorption curve (Fig. 2, extinction coefficient proportional to  $1/\lambda^4$ ). Below this wave-length the thyroglobulin curve increases more rapidly than the serum globulin curve owing to the occurrence of  $K_i$  in the thyroglobulin curve.  $K_g$  is only of importance below 320 m $\mu$  [Gróh & Hanák, 1930]. The value of  $K_g$  is the same at pH 8 and at pH 1.3.  $K_g$  increases first at pH values above 10 [Stenström & Reinhardt, 1925, 1]. Now we can write that at pH 8:  $K_t = K_s + K_i + K_g$  and at pH 1.3:  $K_t' = K_s' + K_i' + K_g'$ . Because  $K_g = K_g'$  we can deduce that

$$K_t - K_t' = (K_i - K_i') - (K_s - K_s').$$

The left-hand member of this equation represents the differential absorption  $\delta_i$  of the iodine compounds. The values of  $K_t$  and  $K_t'$  present in the right-hand member are determined experimentally.

$K_s$  and  $K_s'$  are only known at wave-lengths above 370 m $\mu$  (here  $K_i$  and  $K_g$  are both zero, the absorption is only due to  $K_s$ ). At those wave-lengths  $K_s = K_t$  and  $K_s' = K_t'$ . By application of the Rayleigh-Jeans formula, we can find the values of  $K_s - K_s'$  below 370 m $\mu$  by extrapolation. If these values are calculated by extrapolation of the value at 380 m $\mu$ , which value is denoted by  $(K_t - K_t')_{380}$ , the formula for  $K_t - K_t'$  or  $\delta_i$  becomes

$$\delta_i = (K_t - K_t') - (K_t - K_t')_{380} \times (\lambda/380)^4.$$

In the deduction of this formula we have assumed that pH 8 and pH 1.3 lie outside the interval from  $pK - 2$  to  $pK + 2$ . That the assumption is sound was verified by the measurement of  $K_t$  at different pH values (pH 8.0, 6.8, 6.0) and calculation of the values of  $K_t - K_t'$ . From these data a value between 5 and 6

was deduced for the  $pK$  value of the iodine compounds in thyroglobulin by means of a well-known formula from the theory of dissociation. Therefore  $pH$  8 and 1.3 lie on either side of  $pK - 2$  and  $pK + 2$ .

By means of equation (1) the  $\delta$  curve of the iodine compounds present in thyroglobulin was deduced from the absorption curves of solutions of sheep thyroglobulin (for the preparation of solutions see below under (b)).

The shape of these  $\delta$  curves agreed fairly well with the curves which could be calculated if half of the iodine in the thyroglobulin solution was attributed to thyroxine, the other half to diiodotyrosine. Moreover, the values of the total iodine per ml. of solution which were obtained from the  $\delta$  curves did not differ much from the values which were obtained by direct chemical analysis:

	1	2	3	4	5	6
Iodine per ml. of solution : $\delta$ curves	36	38	36	45	47	49
Iodine per ml. of solution : chemical analysis	38	42	39	47	49	52

1, 2, *Elityran* (a liquid thyroglobulin preparation of I.G. Farbenindustrie) after extraction with ether; 3, own preparation of thyroglobulin; 4, 5, 6, thyroglobulin I.G. Farbenindustrie.

The  $\delta$  curves of the iodine compounds in thyroglobulin which were measured for two solutions (of different samples of thyroglobulin) are reproduced in Fig. 4, curves *A* and *B*. The error in the  $\delta$  values is 3% (error in extinction 1.5%). Accidentally the  $\delta$  curves coincide at  $320 m\mu$ . Now these curves must be compared with the  $\delta$  curves which can be calculated for a thyroxine-diiodotyrosine mixture containing two diiodotyrosine molecules to one thyroxine molecule (iodine equally distributed over thyroxine and diiodotyrosine). This calculated curve is represented in Fig. 4, curve 2. Besides the condition mentioned (thyroxine-iodine = diiodotyrosine-iodine), the total quantities of thyroxine and diiodotyrosine are chosen in such a way that the calculated curve coincides at  $320 m\mu$  with the experimental curves.

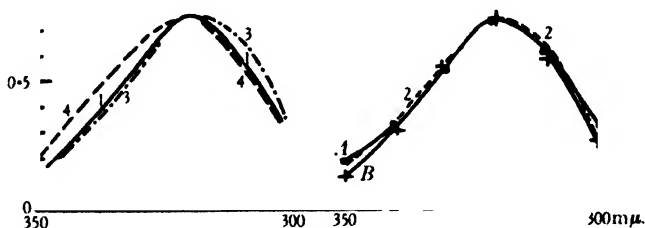


Fig. 4. Experimental  $\delta$  curves (*A* and *B*). Mean values of *A* and *B* (curve 1). Theoretical curve based on ratio of thyroxine-iodine to diiodotyrosine-iodine of 1 : 1 (curve 2); 4 : 6 (curve 3); 6 : 4 (curve 4).

It may be seen that the agreement between calculated and experimental curves is satisfactory. If we compare the total iodine per ml. of solution, which can be deduced from the curve, with the chemical values (as has been done above for six other solutions) we find  $55 \mu g.$  per ml. of solution from the  $\delta$  curve whereas the chemical values are  $55 \mu g.$  and  $51 \mu g.$

In curves 3 and 4 (Fig. 4) the lack of agreement between calculated and experimental  $\delta$  curves is demonstrated if we base the calculation on 40% thyroxine-iodine (curve 3) and on 60% thyroxine-iodine (curve 4). Under these conditions a considerable difference in shape between the calculated and experimental curves (curve 1) is shown. Therefore the value of the diiodotyrosine-



iodine/thyroxine-iodine ratio may be determined with fair accuracy by means of an analysis of the  $\delta$  curve. This ratio cannot differ much from unity in the seven samples of sheep thyroglobulin which have been studied here.

It seems interesting to check whether the same value for this ratio is valid for other kinds of thyroglobulin (ox, hog). As a first experiment the absorption of a solution of ox thyroglobulin was examined (0.7% solution of ox thyroglobulin, I.G. Farbenindustrie).

The experimental  $\delta$  curve agreed with the  $\delta$  curve of a diiodotyrosine-thyroxine mixture which contains 50% thyroxine-iodine. The  $\delta$  curve corresponded to 21  $\mu\text{g}$ . iodine per ml.; the chemical analysis yielded 24  $\mu\text{g}$ . iodine per ml.

These data do not agree with experiments of Leland & Foster [1932], or of Abelin [1932] who found a mean value of about 0.4 for the ratio diiodotyrosine-iodine to thyroxine-iodine in thyroglobulin. Our results seem to agree better with determinations of thyroxine-iodine which have been carried out by Harington & Randall [1929], and Rotter & Mecz [1932]. Rotter & Mecz found a mean value of 1 for the diiodotyrosine-iodine/thyroxine-iodine ratio (19 different thyroglobulin preparations). However, their values vary from 1.4 to 0.4. Our values do not show such variations. This may be explained by the relatively small number of samples (8) which have been studied by us, but will be verified in further experiments.

Here the disadvantage of chemical methods used for the determination of the thyroxine content of thyroglobulin, i.e. changes of the chemical structure, is avoided. On the other hand, the absorption method can only be used for thyroid preparations which yield a clear solution at pH 8 and pH 1.3. The extinction coefficient at 400  $m\mu$  is below 0.7 if iodine concentration is 20  $\mu\text{g}$ . per ml. of solution.

We may conclude that this spectrographic analysis offers strong support for the conception that  $-\text{C}_6\text{H}_2\text{I}_2\cdot\text{O}\cdot\text{C}_6\text{H}_2\text{I}_2\text{OH}$  groups (chromophoric group of thyroxine) and  $-\text{C}_6\text{H}_2\text{I}_2\text{OH}$  groups (chromophoric group of diiodotyrosine) exist in the thyroglobulin molecule. The absorption is mainly due to these chromophoric groups and not to the whole molecule; hence we must not deduce the occurrence of thyroxine and diiodotyrosine as such in the thyroglobulin molecule; changes in the side chains of these iodine compounds remain possible.

Some attention may be paid to the small difference in shape between the experimental and calculated curves. The accuracy of most of our measurements of the  $\delta$  curve have a mean error of 6%. However, the experimental curves A, B of Fig. 4 were determined with the utmost care (see below under d). Therefore we must accept the reality of the small differences between these curves and the theoretical curve (curve 2, Fig. 4).

#### SUMMARY

1. An analysis of the thyroglobulin absorption curve in the region from 400 to 300  $m\mu$  is given. In this analysis differential extinction coefficients have been used. This quantity is defined as the difference between the extinction of the solution if practically all the absorbing molecules have the alkaline form (pH above  $pK+2$ ) and the extinction of the solution if practically all the absorbing molecules occur in the acid molecule-form (acid absorption, pH below  $pK-2$ ).

2. The differential absorption curve of the iodine compounds, as deduced from the absorption curves of (sheep) thyroglobulin at pH 8 and 1.3, gives strong evidence for the presence of the  $-\text{C}_6\text{H}_2\text{I}_2\text{OH}$  group (chromophoric group of diiodotyrosine) and the  $-\text{C}_6\text{H}_2\text{I}_2\cdot\text{C}_6\text{H}_2\text{I}_2\text{OH}$  group (chromophoric group of

thyroxine) in the thyroglobulin molecule; two of the former groups occur to one of the latter groups.

These experiments were suggested by Prof. I. Snapper, to whom the author wishes to express his sincere thanks. He is also indebted to the laboratory staff, especially to Dr A. Grünbaum. Expenses were met by a grant from the Rockefeller Foundation.

# APPENDIX

(a) Determination of the acid absorption of thyroxine. A first attempt to determine the acid thyroxine absorption directly failed (absorption tube of 120 cm. filled with saturated thyroxine solution in 0.1 *N* HCl). Therefore we made use of the adsorption of thyroxine on to a substance which remained soluble at *pH* 1.3.

Dr W. M. Bendien in this laboratory has shown that such a protective colloid is present in serum (horse, human, sheep). He found that thyroxine which is perfectly ultrafiltrable in its solution in 0.02 *N* NaOH becomes non-ultrafiltrable if dissolved in human serum; evidently thyroxine is adsorbed by one of the constituents of the serum (? globulin). With this fact in mind it was easy to get (adsorbed) thyroxine into acid solution.

2 ml. horse serum are mixed with 8 ml. of a 0.066% thyroxine solution (0.02 *N* NaOH). Then 0.3 ml. of *N* HCl is added. In this way the solution is brought to *pH* 1.8 without any precipitation of thyroxine. By subtracting the absorption of a mixture containing 2 ml. horse serum, 8 ml. of 0.02 *N* NaOH and 0.3 ml. of *N* HCl from the absorption of the former solution, the absorption of the thyroxine in acid solution was obtained. Adsorbed thyroxine and free thyroxine show the same absorption, as could be verified in alkaline solution: no appreciable difference could be found between the absorption of a thyroxine solution in 0.02 *N* NaOH and the absorption of a thyroxine solution in alkaline serum.

(b) Thyroglobulin is not easily dissolved if directly treated with acid. In order to obtain an acid solution, the thyroglobulin must be dissolved first in an alkaline solvent. The borate buffer solution of *pH* 8 may not be used as a solvent: if this solution is made acid by adding *N* HCl solution, a small part of the thyroglobulin is precipitated. This is not the case if the thyroglobulin is dissolved in 0.02 *N* NaOH. Thus thyroglobulin solutions at *pH* 8 and 1.3 which may be used for the absorption analysis are obtained by first preparing a solution of thyroglobulin in 0.02 *N* NaOH (about 8 mg. thyroglobulin per ml.). One part of this solution is acidified by adding quickly *N* HCl till the *pH* reaches 1.3, whereas to the other part concentrated buffer-solution is added to bring the *pH* to 8.

(c) The photographic method which was used for the determination of the absorption curves (except those reproduced in curves *A* and *B*, Fig. 4) has been described previously [Ginsel, 1936]. Because this method is based on the use of density marks, the absorption coefficients can be determined at any given wavelength. Its simplicity is due to the fact that only those transparencies of the photographic plate are used which correspond to the straight part of the transparency curve (transparency is intensity of light transmitted by exposed area divided by intensity of light transmitted by unexposed area: transparency curve is transparency plotted against log of relative intensity).

It is stated [Ginsel, 1936] that transparencies between 0.35 and 0.65 correspond to the straight part of the transparency curve. After a longer experience with

Ilford Auto filter plates (400 H and D) we have found however that small deviations from straightness may occur sometimes in this transparency region. The only part of the transparency curve which shows perfect straightness in all the cases is located between transparencies 0.45 and 0.65.

If we are limited to the use of this transparency region, it is only possible to obtain a complete absorption curve if the thicknesses of the liquid layers increase by a factor not greater than 1.7. Unfortunately our tubes increase by a factor 2. Therefore we could not avoid the use of transparencies lying outside the range 0.45–0.65. In this way small errors (below 3%) may have been introduced in most of our absorption curves.

In the determination of curves *A* and *B*, Fig. 4, we did not use the simple method developed by us but reverted to the actual use of density curves, in order to reduce sources of errors. The absorption coefficients were deduced from the densities on the photographic plate in a way similar to the well-known procedure used in photographic intensity measurements.

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# LV. AN ENZYME OF THE TUBERCLE BACILLUS<sup>1</sup>

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*(Received 4 October 1938)*

THE possible importance of bacterial enzymes in the diseases of man has not received the attention it deserves. Particularly in a chronic disease such as tuberculosis the bacterial enzymes, disseminated slowly but with cumulative effect, may well play a decisive role. The study which follows deals with an oxidizing enzyme produced by the tubercle bacillus.

The findings of four groups of workers are in direct conflict in regard to the presence of an indophenol oxidase in the tubercle bacillus. Gordon & McLeod [1928], using *p*-aminodimethylaniline (*p*-dimethylphenylenediamine) alone, reported a 1 plus result for the tubercle bacillus on a scale where the *meningococcus* was 4 plus, confirming the 1 plus result of Schultze [1910] and Kramer [1912] who used the diamine in combination with  $\alpha$ -naphthol. But Terada & Nozaki [1937], in a study concerning human and rat lepra bacilli and the other acid-fast organisms, using the diamine alone as did Gordon & McLeod, found no indophenol oxidase in either the pathogenic or non-pathogenic acid-fast bacilli, while their controls of *B. subtilis* and *meningococcus* gave the proper positive results. Kawabata [1934] also reported negative results for indophenol oxidase in human, bovine and frog tubercle bacilli.

No reference has been found in the literature to the presence in the tubercle bacillus of a catechol or phenol oxidase, but a catechol oxidase frequently occurs in organisms producing an indophenol oxidase as shown by Happold [1930]. The intensities of the enzymic reactions were not parallel for the two, and one organism (*Staph. albus*) possessed the catechol but not the indophenol oxidase.

A search for the enzyme which is the subject of this paper was undertaken because it appeared that some of the properties of a phenolase or catechol oxidase might explain certain observations made in the laboratory regarding pigmentation in tuberculous tissue, and the reddening and darkening of masses of tubercle bacilli which had been left for some weeks in a phenol solution. The work described is preliminary to a more extended study now under way.

## EXPERIMENTAL

### *Testing of bacillary masses with phenolic substances*

The original observation regarding the darkening of masses of bacilli was verified by placing equivalent masses of tubercle bacilli of human type in duplicate bottles of 5 % phenol, sterile physiological saline and sterile distilled water: one trio was kept in the dark and the other was exposed to the usual laboratory lighting. The bacillary masses changed colour only in phenol, and the reddening occurred sooner and darkened more in those exposed to the light. In all subsequent tests the materials were accordingly exposed as evenly as possible to light.

<sup>1</sup> Presented in part before the Division of Medicinal Chemistry at the Ninety-Fourth Meeting of the American Chemical Society, 10 September 1937, at Rochester, New York.

*Variation of phenol concentration.* Aqueous solutions were used. Ten concentrations, from 0.5 down to 0.1 %, and from 0.05 to 0.01 % respectively, were tested in 5 ml. portions. The human type bacillus (Gary strain) was used; one culture had been recently planted and the other was an old one, suspected of being dead. Comparable crumbs of bacilli (20–40 mg.) were removed from the solid medium and placed in the solutions with a nichrome wire loop.

Reddening of both organisms was observed in 1 day in the 0.5, 0.4 and 0.3 % tubes. Both reddened in 2 days in the 0.2 % phenol. The freshly recovered strain reddened in the 0.1 % tube in 4 days, but not the old culture. None of the 0.05–0.01 % tubes gave any coloration within 2 weeks. For practical purposes, therefore, it would be advisable not to use a lower concentration of phenol for testing than 0.2 %.

An upper limit for the phenol concentration cannot be determined. Homogeneous mixtures of phenol and water cannot be obtained between approximately 10 and 70 % phenol. Human type bacilli (Gary), taken off solid medium, were tested in 90, 80 and 75 % phenol. Swelling and some dispersion of the crumbs occurred, but no darkening within 12 days. Five months later complete solution of the bacillary masses was the only change to be observed.

*Phenol in phosphate buffer.*  $\text{KH}_2\text{PO}_4$ -NaOH mixtures, made up according to Clark [1925] at pH 5.8, 6.0, 6.2, 6.4, 6.6, 7.0 and 7.4, containing 5 % phenol, were used in 5 ml. portions with equal-sized crumbs of human bacilli (Gary) freshly grown on 5 % glycerol broth. In 1 day a faint, shrimp-pink tint was seen on the edges of the crumbs in all the tubes. After 3 days the colour had spread throughout the bacillary masses, that in the pH 6.0 tube being the darkest; next day there was a suggestion of dark speckling in this tube. Two months later only a uniformly deep rose colour appeared in all the tubes, somewhat greyer in tinge than the original.

#### *Preliminary trials with substances other than phenol*

*Orcinol*, *thymol* and *p-benzoquinone* were tested in saturated aqueous solution; *catechol* and *resorcinol* were used in 1 % aqueous solution. Crumbs of the same human type bacilli (Gary), used above for the phenol-phosphate tests, were used here, in 5 ml. of the solutions of which another 5 ml. were kept as a control in which colours developed by autoxidation could be seen.

There was no immediate change in any tube except that containing the quinone, where a deep red-brown developed in the solution around the bacillary masses; in 22 hr. the flakes were deep brown and the solution itself darker than the control. At 22 hr. the catechol solution containing bacilli was pink, the control colourless; the orcinol test mixture was slightly pinker than its control. After 48 hr. the quinone-coloured flakes were very dark, those in the catechol were reddish grey (with the autoxidation control still colourless), and those in orcinol had faint pink edges. After 7 weeks the quinone mixture was dark and very turbid, preventing further description; the bacilli had become brownish black in catechol (while the control was only a weak tea-colour); in orcinol the masses were a rosy, greyish brown, the control a light brownish red. Resorcinol and thymol solutions remained negative throughout, no colour developing with or without bacilli.

In connexion with the colour developed in the *p-benzoquinone*, curiosity prompted the filtration of some of the culture fluid from this same lot of bacilli through a Berkefeld grade N candle, and the testing of the filtrate with quinone. A deep brownish red colour developed instantaneously and became more intense overnight. A portion of sterile glycerol broth of the same batch used for the culture was also tested with quinone and gave qualitatively similar results but

very much more slowly. Presumably substances capable of interaction with quinone had been added to the medium during bacillary growth. It should be noted that the bacilli themselves became darkened in the presence of quinone, and while this might mean further oxidation of quinone by enzymic means, it might mean a localized combination of quinone with bacillary substance, which was dark in colour.

*Phenol, catechol, resorcinol, quinol, pyrogallol, hydroxy-quinol*  
(1:2:4-trihydroxybenzene), *phloroglucinol, tyrosine, adrenaline*

Phosphate buffer at pH 6.6 was used to prepare 1% solutions of all the phenol family, and a saturated solution of tyrosine: a 5 ml. portion of each solution was used for the test, and 5 ml. used as a control for observation of colour developing with autoxidation; the adrenaline used was the commercial 1:1000 solution of the hydrochloride, containing preservatives. Crumbs of equal size of human type bacilli (strain Gary), grown on solid medium, were placed in the test solutions.

At this pH (6.6), during 44 hr. observation, the phloroglucinol, resorcinol, phenol and tyrosine tests were all negative. Results with the other compounds follow. 1:2:4-Trihydroxybenzene: the control itself darkened too readily to permit observation of the bacilli, but they were already very dark at 8 hr. Pyrogallol: the bacillary mass was orange at 8, very brown at 31 and black at 44 hr. Quinol: a questionable coloration of the mass was noted at 8 hr., which had deepened to a definite rose at 31, and to a dark reddish brown at 44 hr. Catechol: the bacillary mass was brownish at 8 hr., and the brown colour became progressively more intense at 31 and 44 hr. Adrenaline: no colour change had been observed at 44 hr., but in view of the preservatives present (chlorotone 2½ grains per ounce, and sodium bisulphite not over 0.1%) the tube was kept for further observation: after 27 days the bacillary mass had become a brownish grey, which was still darker after 6 more days. In the absence of preservatives the adrenaline would be expected to show an activity similar to that of catechol; the concentration of the adrenaline was also only one-tenth that of the catechol.

From the respective colorations of the bacillary masses with passage of time, one would derive the following order of diminishing oxidizability (diminishing reducing power) at pH 6.6: 1:2:4-trihydroxybenzene, pyrogallol, catechol, hydroquinone, adrenaline (with preservatives present), and phenol (other long-term experiments with Gary cultures).

#### PRODUCTION OF DIANILINO-O-BENZOQUINONE

Because of the several chemical manipulations necessary, the non-virulent type of tubercle bacillus (BCG) was chosen for the first experiment, which followed substantially the preparation of dianilino-o-quinones by Pugh & Raper [1927] and by Happold [1930] using enzymes from various sources. Sterile apparatus was used throughout.

A suspension of BCG was prepared by grinding crumbs of bacilli with physiological saline in an agate mortar; the 35 ml. of suspension used for the experiment contained 105 mg. of bacilli. The suspension was added to 100 ml. of Clark's  $K_2HPO_4$ -NaOH buffer at pH 6.5, containing 1 g. catechol and 2 ml. aniline. No immediate colour developed after mixing, and the preparation was allowed to stand overnight, free access of air being provided through the sterile cotton plug in the flask. After 16 hr. a red precipitate with a somewhat brownish tinge appeared, consisting of just-visible tufts of fine short needles; the liquid

was coloured red also. The flask was aerated for 2 hr. with gentle bubbling, first passing the air through chloroform-water, and a marked increase in the amount of precipitate resulted.

A few drops of the mixture, containing some precipitate, were examined microscopically. Beautiful garnet red bundles of needles were seen among considerable débris; feather and burr forms and single needles were all present. The red crystals in the precipitate were extremely friable; a portion of the mixture was filtered, but the crystals could not be removed from the filter paper without admixture with paper fibres; the filter paper was dried over sulphuric acid. The M.P. of the crude material, still containing bacilli and débris, was 186.5° (uncorr.).

Of all preparations of dianilino-*o*-benzoquinone made subsequently, the one to be described next was the most successful.

#### *Culture fluid with catechol and aniline*

The culture fluid (75 ml.) from a 3 months' growth of human type bacillus (Gary) upon 5% glycerol broth was decanted from bacillary sediment after centrifuging. To it was added an equal volume of phosphate buffer at pH 6.5, containing 1 g. catechol and 2 ml. aniline.

A faint pink colour developed immediately, rapidly darkening in tint; the reaction mixture was red and a few red needles had already formed in 15 min. In 5 hr. a heavy precipitate had settled, which was increased in bulk on standing overnight; the liquid still contained much red material in suspension. This preparation was not aerated at any time, but absorbed the necessary oxygen at its surface. (Owing to the virulence of this strain, whose bacilli might be present in the precipitate, no chemical manipulation of the product was attempted.)

The control flask, made up of 75 ml. (sterile) of the same lot of glycerol broth on which the culture had been grown, with the same buffer, catechol and aniline, produced a small amount of dirty light brown flocculent material after an hour; this settled out overnight, leaving a clear light brown liquid; no red crystals were seen at any time during the 18 hr. the mixture was under observation. It is therefore clear that the growth of tubercle bacilli in this broth had produced an agent capable of very marked catalysis of the oxidation of catechol, as manifested by the rapid and voluminous formation of the dianilino-derivative of its oxidation product, *o*-benzoquinone.

Since the result of using decanted culture fluid instead of a bacillary suspension was so satisfactory, the next step was to try a fluid which could not be suspected of containing any bacilli whatever.

#### *Berkefeld filtrate from human type bacilli*

The culture fluid from a growth of the virulent strain H 37 upon 5% glycerol broth was passed through a grade N Berkefeld candle and tested with the usual proportions of catechol and aniline in phosphate buffer at pH 6.5. A very marked catalysis of the oxidation of catechol was observed.

Further possible avenues of approach to a study of bacillus-free preparations of the enzyme were thus opened.

*Note.* Another preparation of the dianilino-benzoquinone was made, using a sterile Berkefeld filtrate from a culture of the non-virulent BCG. The red crystals, after decanting the reaction mixture, were washed once, centrifuged and spread on a clay plate. The M.P. of this still relatively crude product (air-dried for 3 days) was 190°, with decomposition, which compares favourably with 193°, the value reported by Happold for his most highly purified product.

*Effect of bacillary suspension on phenol, guaiacol, orcinol and quinol  
in presence of aniline*

A suspension of washed human type bacilli (Gary) was used for all these experiments. Approximately 50 mg. of bacilli were used for each 50 ml. of phosphate buffer at pH 6.5, containing the usual proportions of the phenol in question, and aniline.

None of these phenols formed the red dianilino-*o*-benzoquinone. No visible product appeared in the guaiacol mixture; brownish yellow substances were observed in the phenol, orcinol and hydroquinone mixtures.

The failure to obtain evidence of the formation of an *o*-quinone in the case of orcinol agrees with the conclusion of Happold [1930] concerning the oxidation of that substance, and in the cases of phenol and guaiacol it may perhaps be considered additional evidence that the mode of action of the enzyme under discussion is not that of tyrosinase [Pugh & Raper, 1927].

*Old tuberculin, long-dried bacilli*

It seemed of interest to investigate the retention of this particular enzymic activity in old tuberculin (which is essentially a concentrated sterile filtrate of culture broth from the human type bacillus), and in a stock of dried human type bacilli (H 37) which had been kept over sulphuric acid for approximately 10 years.

10 ml. of old tuberculin (representing 100 ml. of fresh culture fluid) were mixed with 90 ml. of distilled water and 100 ml. of phosphate buffer at pH 6.5, containing the usual 1 g. catechol and 2 ml. aniline. The weight of the dried bacilli used, 0.54 g., was approximately five times that used in the fresh bacillary suspensions previously tested; the bacilli were suspended in 80 ml. buffer at pH 6.5, containing 0.75 g. catechol and 1.5 ml. aniline. In this case an autoxidation control of the reagents was kept, since it was expected that observations would continue for a long period. (The control of sterile broth used with the decanted culture fluid-catechol experiment was also available for comparison with the old tuberculin.) After 2 months so much brownish deposit had formed that it was useless to try to weigh the yields of red crystals of the dianilino-*ortho*-benzoquinone, and only visual comparison was possible.

There appeared to be little difference between control and dried bacilli. Apparently the enzymic activity had been lost during the ageing and drying of the bacilli.

The old tuberculin mixture, however, showed quite definitely a heavier yield than the controls. This particular preparation thus had seemingly retained some of its oxidase activity, but whether this represented the total activity of the old tuberculin after manufacture could only be decided by tests at repeated intervals of storage.

ATTEMPT TO USE METHYLENE BLUE AS HYDROGEN ACCEPTOR IN THE  
OXIDATION OF CATECHOL

It was thought that if methylene blue could be utilized as hydrogen acceptor in place of molecular oxygen, a quantitative method for estimation of the enzyme could be worked out, using catechol as the substrate and depending on the rate of decoloration of the dye. An attempt was accordingly made to form the dianilino-*o*-benzoquinone from catechol (as evidence of oxidation to the quinone) in the presence of methylene blue, with air excluded from the reaction by a deep layer of paraffin oil.



The test mixture contained 2 ml. of a thin suspension of human type bacilli (H 37) ground in physiological saline, 3 ml. of 1 : 5000 methylene blue solution, 5 ml. of phosphate buffer at pH 6.5, 0.05 g. catechol, 1 drop of aniline; after thorough mixing the liquid was covered with 2 in. of sterile paraffin oil. A control with 2 ml. distilled water in place of the bacillary suspension, and a control containing only buffer, methylene blue and aniline, properly diluted, were also prepared and covered with oil.

No reduction of methylene blue occurred during the 7 days the tubes were under observation. After 3 days there was evidently some leakage of air through the oil layer, for in the tube containing bacilli a few red crystals formed very slowly at the oil-water interface. At pH 6.5 and 23° methylene blue did not serve as hydrogen acceptor in this reaction.

#### THERMOSTABILITY OF THE ENZYME

The supernatant fluid from a centrifuged culture of human type bacilli (H 37), grown on 5% glycerol broth, was used for the tests in place of a Berkefeld filtrate, in order to avoid dilution from a steam-sterilized candle and any possible physico-chemical effects of the material making up the candle. Sterile graduated centrifuge tubes, selected for uniform shape and size of tip, were used to heat the fluid, of which 2 ml. were used for each test. The tube was clamped over a water bath so that the fluid was well immersed, heated for  $\frac{1}{2}$  hr. at the selected temperature, and then stored immediately in a mixture of ice and water. An unheated tube was stored in the ice as control. The temperatures used were 45, 55, 65, 75, 85 and 98°.

In this experiment the formation of dianilino-quinone was used as a test for any enzymic activity remaining. To each of the chilled tubes 2 ml. of phosphate buffer at pH 6.6, containing 1% catechol and 2% aniline, were added, and a chemical control tube with no culture fluid was also made up. The tubes were allowed to stand with sterile cotton plugs to admit air. The only observable difference 4 hr. after mixing was the slightly darker colour of the heated tubes compared with the unheated control; 17 hr. after mixing there were a few amorphous reddish bits to be seen in the culture fluids, but none in the control. In another 24 hr. there were clumps of definite crystals of red dianilino-o-benzoquinone in all the culture fluids, while there was only the merest suspicion of red material in the chemical control. There was no differentiation between tubes heated to different temperatures, or between heated and unheated controls, at this stage. (Although this was not a potent fluid compared with most of those used in the foregoing experiments, it still caused the appearance of an effect in 17 hr. which required 41 hr. to manifest itself unaided.) After another 48 hr. (89 all told) there were still very few crystals in the chemical control, but a moderately heavy red precipitate in all the culture fluids. The tubes were centrifuged to pack the sediment evenly in the graduated tips. There was still no obvious difference to be seen between the test temperatures, or between heated and unheated fluids. It was concluded that the enzyme is thermostable, contrary to expectations. Various plant polyphenolases have been reported as thermostable, according to Haldane [1930].

#### SEPARATION FROM CULTURE FLUID OF A PROTEIN POSSESSING ENZYMIC ACTIVITY

The amount of red dianilino-quinone formed, again served as a rough measure of the enzymic activity, using catechol as substrate. The fluid used was that decanted after centrifuging a 5% glycerol broth culture of human type bacilli

(H 37). Freshly prepared 50% trichloroacetic acid was used as protein precipitant, in the proportion of 1 vol. acid to 4 vol. fluid, as used by Seibert [1934] at a certain stage in the preparation of the tuberculo-protein known as P.P.D. The protein in four 5 ml. portions of fluid was thrown down and allowed to settle overnight. After being centrifuged the tubes were allowed to stand another 24 hr.; a light secondary coagulum formed, which was adherent to the walls of the tubes and was not affected by centrifuging. From two of the tubes the supernatant fluid was decanted, and the precipitated protein stirred up well with 5 ml. of freshly prepared 10% trichloroacetic acid, centrifuged, the wash-liquid discarded and the washing with 10% acid repeated. Then the protein was dissolved carefully in the least possible amount of *N*/10 NaOH, 1.5 ml. being required for this lot, and made up to 5 ml. with distilled water. The originally decanted supernatant fluids were carefully neutralized with 10% NaOH (to dilute them as little as possible, instead of using *N*/10 NaOH). The other two tubes, still containing protein and original reaction mixture, were likewise neutralized approximately with the strong alkali, and finally with the weak; these were to serve as controls of the possibly destructive effect of trichloroacetic acid upon the total enzyme content of the fluid. A control of neutralized trichloroacetic acid itself was also prepared. To each of these (redissolved protein, neutralized supernatant fluid, neutralized whole reaction mixture and acid control) and also to portions of the original culture fluid, an equal volume of phosphate buffer at pH 6.6 was added. The buffer had 1% of catechol and 2% of aniline already dissolved in it. Sterile cotton plugs permitted absorption of oxygen as usual.

After 21 hr. it was apparent from the relative volumes of precipitated dianilino-quinone that part of the enzymic activity had been carried down with the protein precipitate, and part had remained in the supernatant fluid. After another 24 hr. the dark red crystals were packed down by centrifuging and the amounts in the tubes compared. The precipitated, twice washed, redissolved protein contained about three-fifths of the original activity, while two-fifths remained unprecipitated and still active in the supernatant reaction mixture. The enzymic activity had apparently not been damaged by trichloroacetic acid, since the neutralized whole reaction mixture had as much dianilino-quinone formation as the untreated original culture fluid. As usual, the few crystals formed in the autoxidation control were negligible in amount.

#### SUMMARY

Various experiments have been described wherein the human type of tubercle bacillus or a product of its growth was found to catalyse the oxidation of various phenolic substances. The properties of the active agent concerned in this catalysis, as observed under the conditions employed, suggest the formation of a diffusible, thermostable enzyme of the polyphenol oxidase type, whose behaviour is more consistent with catechol- than with phenol-oxidase, and which is not a tyrosinase. Part of the activity could be removed from a culture fluid of human type tubercle bacillus by use of a protein precipitant, and recovered from a solution of the washed protein. Methylene blue did not serve as a hydrogen acceptor for the oxidation of catechol catalysed by this enzyme.

The author wishes to thank the Organic Chemicals Division of the Eastman Kodak Company for the preparation and gift of 50 g. of 1:2:4-trihydroxybenzene, without which a complete study of the phenol family could not be made.

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# LVI. THE METABOLISM OF LACTIC AND PYRUVIC ACIDS IN NORMAL AND TUMOUR TISSUES

## VI. OX RETINA AND CHICK EMBRYO

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IN rat and rabbit kidney cortex slices, lactate is oxidized to pyruvate and the greater part of the pyruvate is removed by a cyclic series of reactions whereby 2 mol. of pyruvate are converted successively into succinate, fumarate, malate and oxaloacetate; the oxaloacetate then decomposes spontaneously to yield pyruvate in half the original amount; in tumour, brain, testis and liver, however, this cycle does not occur to any extent [Elliott & Schroeder, 1934; Elliott *et al.* 1935; 1937].

The present investigation deals with the metabolism of lactate and pyruvate by ox retina and chick embryo. The same manometric and analytical methods were used as for previous studies of this series [Elliott & Schroeder, 1934; Elliott *et al.* 1935]. Some possible intermediates, not tested with other tissues, were also used as substrates in an attempt to elucidate the mechanism of the breakdown of lactate and pyruvate.

### EXPERIMENTAL

The concentrations of substrates in the medium were: *dl*-lactate *M*/25; pyruvate, acetate,  $\alpha$ -ketoglutarate,  $\beta$ -hydroxybutyrate, formate *M*/50; fumarate, *l*-malate, oxaloacetate, citrate, oxalate *M*/100; succinate *M*/100 and *M*/25. The experimental period, except where otherwise stated, was 90 min. The terms defined in previous communications of this series were adopted for expressing results.

In these experiments manometers with a wider bore and Clerici fluid containing only thallium malonate and thallium formate were used as suggested by Dixon [1937]. These changes decreased the errors of the apparatus, so no corrections of the observed  $Q_{CO_2}$  and R.Q. values have been made.

The chemical analyses for lactate, pyruvate and oxaloacetate were carried out as described by Elliott *et al.* [1935]. Elliott *et al.* [1937] concluded from work with liver that the method previously used [1935] for the estimation of oxaloacetate as keto-body was unsatisfactory in the presence of tissue materials. However, the unexpectedly low results obtained by them were probably correct and were due to the rapid reduction by the tissue of part of the oxaloacetate to malate [see Stare, 1936; Elliott & Elliott, 1939]. The earlier method for the estimation of oxaloacetate was therefore employed.

Only typical results, representative of a large number of experiments, are given in the tables.

## OX RETINA

Ox eyes were obtained from the abattoir on the morning of slaughter, but it was impossible to determine the exact time of killing. Some variations in results, therefore, were possibly due to the fact that the tissue was not as fresh as desired for some experiments.

For 4 manometers the retinas of 4 eyes were used, the eyes of each pair being considered identical for purposes of sampling. The cornea, iris, lens and vitreous humour were removed and the retina, while still in place in the resulting capsule, was divided radially from the optic nerve with a scalpel and taken out in sections. The tissue was kept in Krebs's medium containing glucose through which  $O_2/CO_2$  was bubbled. After rinsing in Ringer-Locke solution (this was done twice for experiments in the absence of glucose), the material for each vessel was drained on perforated porcelain discs over filter paper and weighed. The nasal side of the retina (C in Table I) usually had a somewhat lower respiration and glycolysis than the temporal side (A and B), so for the experimental vessels roughly equivalent weights of retina from the temporal side of each pair of eyes were used. The nasal side was used in the left-hand vessels. In some preliminary experiments, the sections of retina from the temporal side were divided parallel to the circumference (the sections nearer the optic nerve were designated as A and those nearer the circumference as B); rather wide differences in  $Q_{O_2}$  were found though the differences were not consistent. When similar radial samples of the whole temporal side were taken, variations in metabolism were smaller.

Table I. *Ox retina metabolism. Comparison of different sections*

Glucose in medium, no other substrate added										
	A	B	C	A	B	C	A	C	A	B
$-Q_{O_2}$	10.6 (20.8)	12.1 (22.3)	10.7 (20.0)	10.6 (21.2)	13.4 (23.9)	8.8 (19.5)	8.1 (20.6)	6.7 (16.6)	10.0 (22.6)	8.8 (17.8)
R.Q.	0.83	0.88	0.86	0.76	0.79	0.93	0.95	0.93	0.89	0.92
$Q_A$	11.6	10.5	10.8	11.9	11.4	6.8	7.7	7.0	7.4	7.9

Figures in parenthesis are calculated on the basis of final dry wts.

In all cases the results were calculated on the initial dry weight, i.e. on the dry weight deduced from the wet weight taken and the wet weight/dry weight ratio of a separate sample. In some experiments values for  $Q_{O_2}$  were also calculated on the basis of the final dry weight, but these were not reliable since the tissue disintegrated to such an extent, especially after the acid was added, that it could not be removed completely from the medium.

Like brain, retina exhibits in the presence of glucose a very high initial glycolysis which falls off rapidly. This is shown by the slope of the composite curve for the experiment [see Elliott *et al.* 1937, p. 1004] and by the considerable amount of lactate formed in the left-hand vessel during the 20 min. of gassing and equilibration. The  $Q_{LA}$  of ox retina, calculated on the initial dry weights, varies between +22 and +29 for the first 20 min., between +8.5 and +9.9 for 1 hr. experiments, and between +5.1 and +7.6 for the usual experimental time of  $1\frac{1}{2}$  hr.

**Lactate.** Lactate is metabolized by retina at a rapid rate. Part is oxidized completely, as indicated by the increase in  $Q_{O_2}$ , which corresponds roughly to the acid disappearing (1 lactate requires  $3O_2$  for complete oxidation), and part is changed to pyruvate as shown by the increase in  $Q_{keto}$ . When lactate or glucose

Table II. *Ox retina*

Substrate	Glucose	$-Q_{O_2}^*$	R.Q.†	$Q_A$	$Q_{LA}^\ddagger$	$Q_{keto}$
No addition	+	11.9 (22.6)	0.88	+7.5	+6.5	+0.5
No addition	+	12.0 (25.0)	0.92	+5.1	+5.2	(206 mg. wet wt ) +0.1 (403 mg. wet wt )
No addition	-	8.3 (18.7)	0.86	-0.5	-0.1	0
Lactate	-	14.5 (26.6)	0.87	-2.8	-3.8	+1.4
Pyruvate	-	16.5 (32.8)	1.24	-6.2	+2.3	-7.7
Succinate <i>M</i> /100	-	9.3 (13.5)	0.79	-1.2	+0.1	0
No addition	-	6.6	0.95	-0.4	—	—
Lactate	-	10.8	0.87	-2.3	—	—
Succinate <i>M</i> /100	-	6.9	0.80	-0.8	—	—
Succinate <i>M</i> /25	-	8.2	0.66	-0.6	—	—
No addition	+	10.7 (38.1)	0.87	+6.8	+6.4	+0.2
No addition	+	9.1 (37.2)	0.96	+6.9	+6.2	+0.5
Lactate	+	11.2 (24.7)	0.82	+7.1	+4.3	+0.2
No addition	+	11.2	0.80	+8.6	+7.6	0
			(364 $\mu$ l. $CO_2$ ) ( + 355 $\mu$ l. $CO_2$ )			
Pyruvate	+	9.6	1.34	+2.8	+5.3	-4.6
No addition	+	11.3 (22.7)	0.86	+8.3	+7.6	0
Pyruvate	+	14.3 (23.1)	1.17	+1.9	+6.9	-5.0
Acetate	+	14.1 (22.4)	0.82	+7.7	+7.3	-0.2
No addition	-	7.4 (24.8)	0.97	-0.3	0	0
Fumarate	-	8.6 (19.5)	0.89	-0.5	[ +1.2 ]	0
Malate	-	8.0 (20.4)	0.94	-1.4	[ -1.5 ]	+0.3
Acetate	-	8.2 (20.6)	0.94	0.5	-0.5	0
No addition	+	11.0 (21.8)	0.94	+6.8	+5.2	0
Fumarate	+	11.2 (22.0)	0.89	+7.4	[ -6.2 ]	0
Malate	+	11.7 (22.8)	0.87	+6.8	[ +6.3 ]	0
Acetate	+	12.2 (23.0)	0.89	+6.5	+4.7	0
No addition	+	11.3 (20.0)	0.82	+6.4	+5.1	+0.2
Citrate	+	10.4 (18.4)	1.02	+6.1	+5.2	-0.2
No addition	-	6.8 (12.8)	1.01	-1.0	—	—
Citrate	-	7.0 (11.2)	1.03	-1.1	—	—
Formate	-	6.2 (13.3)	0.97	-0.7	—	—
No addition	-	7.5 (17.5)	0.87	+0.2	-0.2	-0.1
			(304 $\mu$ l. $CO_2$ ) ( + 16 $\mu$ l. $CO_2$ )			
Pyruvate	-	13.7 (25.6)	1.12	-3.5	+1.4	-4.9
Oxaloacetate	-	14.5 (28.0)	1.64	-12.4	+1.5	-7.1
			(1033 $\mu$ l. $CO_2$ ) ( - 530 $\mu$ l. $CO_2$ )			
Oxaloacetate, no tissue	-	—	(320 $\mu$ l. $CO_2$ ) ( - 360 $\mu$ l. $CO_2$ )		0	—
No addition	-	7.8 (14.3)	0.96	-1.3	—	—
$\beta$ -Hydroxybutyrate	-	7.7 (13.4)	0.86	-0.2	—	—
No addition	+	10.0 (14.3)	0.85	+9.1	—	—
$\beta$ -Hydroxybutyrate	+	10.3	0.94	+7.8	—	—
No addition	+	8.7	0.81	+7.5	+6.6	0
$\alpha$ -Ketoglutarate	+	9.2	0.93	+6.1	+5.5	+0.2
No addition	-	8.1	0.88	-0.6	—	—
Oxalate	-	8.3	0.82	-0.3	—	—
$\alpha$ -Ketoglutarate	-	7.5	0.90	-0.9	—	—
<i>Anaerobic</i>						
Substrate	Glucose	$Q_{CO_2}$	$Q_A$	$Q_{LA}$	$Q_{keto}$	
No addition	-	0	+1.8	+1.2	-0.1	
Pyruvate	-	0.3	+1.8	0.2?	-1.5	
Pyruvate	-	0.8	+1.7	+1.6	-1.5	
No addition	-	0	+2.7	+1.2	0	
			( + 122 $\mu$ l. $CO_2$ )			
Oxaloacetate	-	7.2	-5.0	+3.9	-4.3	
		(319 $\mu$ l. $CO_2$ )	( - 221 $\mu$ l. $CO_2$ )			
Oxaloacetate, no tissue	-	(276 $\mu$ l. $CO_2$ )	( - 310 $\mu$ l. $CO_2$ )			

\* See Table I.

† The figures for  $\mu$ l.  $CO_2$ , given in parenthesis under R.Q. or  $Q_{CO_2}$ , represent the total respiratory  $CO_2$  evolution during the experimental period. The figures for  $\mu$ l.  $CO_2$  under  $Q_A$  represent the total  $CO_2$  evolved or absorbed as the result of acid changes.‡ Figures in [brackets] under  $Q_{LA}$  refer to estimations given wholly or partly by malate.

is present a small amount of pyruvate is formed in both vessels (*c.* 0.1 mg.), the amount in the left being only slightly less than in the right, indicating a high initial rate of keto-formation and that the path of glucose oxidation is probably through pyruvate. Lactate and glucose appear to be alternative substrates for retina since, in the presence of glucose, lactate has little effect on  $O_2$  uptake or R.Q. However, with both glucose and lactate present there is often some discrepancy between lactate and acid formation, acid formation being greater than lactate formation. This indicates the accumulation of some unknown acid which is not lactate or pyruvate. This phenomenon appears occasionally to a slight extent when glucose alone is the substrate.

In order to determine whether the tissue is damaged and respiration falls off in the absence of substrate [see Krebs, 1935], and to investigate further the unknown acid, experiments of 1 and 2 hr. duration were done. The results of these experiments are given in Table III together with values calculated by

Table III. *Ox retina: 1 and 2 hr. experiments*

Substrate	Glucose	Exp. period	$-Q_{O_2}$ *	R.Q.	$Q_A$	$Q_{LA}$	$Q_{keto}$
No addition	-	2 hr.	6.4 (15.6)	0.94	-0.8	-0.2	0
Lactate	-	2 hr.	12.7 (27.4)	0.97	-3.5	-2.7	+0.1
No addition	-	1 hr.	9.0 (21.8)	0.91	-0.9	-0.6	0
Lactate	-	1 hr.	13.8 (24.3)	0.91	-3.0	-5.3	+0.3
No addition, 2nd hr. by diff.	-		3.8	1.03	-0.6	+0.3	0
Lactate, 2nd hr. by diff.	-		11.7	1.01	-4.0	0	-0.4
No addition	-	2 hr.	8.0	0.94	-0.7	-0.2	-0.3
Pyruvate	-	2 hr.	14.7	1.14	-4.3	+1.3	-6.4
No addition	-	1 hr.	8.1	0.85	-0.2	0	+0.9
Pyruvate	-	1 hr.	14.8	1.22	-5.1	+1.3	-6.3
Lactate	-	2 hr.	15.2	0.93	-4.0	-2.3	—
Lactate + pyrophosphate	-	2 hr.	19.8	0.97	-5.2	-4.4	—
Lactate	-	1 hr.	15.3	0.92	-3.3	-5.0	—
Lactate + pyrophosphate	-	1 hr.	22.5	0.98	-6.3	-8.5	—

\* See Table I.

difference for the second hour. Without substrate,  $O_2$  uptake falls off somewhat with time. With added lactate the  $Q_{O_2}$  is increased and falls off only slightly, with the result that during the second hour the difference between the  $O_2$  uptakes in the presence and absence of lactate is considerably greater than during the first hour. The R.Q., with and without lactate, is usually higher for the second hour than for the first.

In the 1 and 2 hr. experiments, the discrepancies between  $Q_A$ ,  $Q_{LA}$  and  $Q_{keto}$  are much more marked than in the usual 90 min. experiment. During the first hour there is a large lactate disappearance while only a part of this is accounted for by acid disappearance. Since no appreciable amount of keto-body accumulates, the formation from lactate of some as yet unknown acid intermediate is presumed. During the second hour lactate is no longer metabolized but the acid intermediate continues to be oxidized. There is quite a large acid removal,  $Q_A = -4$ ; but no lactate removal,  $Q_{LA} = 0$ . A similar phenomenon appears in the work reported by Elliott *et al.* [1937] in some experiments with liver. In the presence of glucose, the formation and disappearance of the unknown acid still play a part but the effects are more variable.

Since retina is reputed to be one of the richest sources of phosphatase [Reis, 1937], it was thought that the unknown acid might be phosphoric acid.

Phosphate determinations, however, showed that this would not account for the unknown acid. Possenti [1935, 1] also found no liberation of  $\text{H}_3\text{PO}_4$  by retina during glucose fermentation.  $\text{NH}_3$  determinations were also carried out (by Dr J. Harand of the micro-analytical department) but these did not throw any light on the peculiar acid formation and disappearance [see also Rösch & Kamp, 1928; Stutzke, 1936; Dickens & Greville, 1933].

Another possibility was that the unknown acid which accumulated from lactate in the absence of glucose might be some intermediate such as acetate, which, although not oxidized to any extent when present as substrate, might be metabolized if a certain concentration of other metabolite, e.g. pyruvate, were also present. An experiment was done with acetate in the presence of  $M/1000$  pyruvate but this showed no increased removal of acetate.

Pyrophosphate increased the respiration in the presence of glucose or lactate, but unknown acid accumulated in the first hour and disappeared in the second hour as usual (see Tables III and IV). The effects of pyrophosphate are discussed in another paper [Greig & Munro, 1939].

*Pyruvate.* Possenti [1935, 2] found that anaerobically retina produces little or no  $\text{CO}_2$  from pyruvate, but aerobically the  $\text{O}_2$  consumption is markedly increased, the r.q. also being increased from 0.8–0.9 to above 1. Our results agree with these. The rate of pyruvate oxidation is equal to or slightly greater than that of lactate. Part of the pyruvate disappearing is reduced to lactate, the rate of lactate formation being very high at the beginning of the experiment ( $Q_{\text{LA}}$  values for the first 20 min. were +19, +20, +19.7, +22, calculated from the analysis of the medium of the left-hand vessel): part is completely oxidized as is shown by the increased  $\text{O}_2$  uptake, raised r.q. and acid disappearance. The presence of glucose does not seem to affect pyruvate metabolism ( $-Q_{\text{keto}}$ ), but it is difficult to determine exactly its rate of oxidation since a part is reduced to lactate, and lactate is also formed by glycolysis. Anaerobically there is negligible decarboxylation.

One- and two-hour experiments, analogous to those with added lactate discussed above, were done using pyruvate as added substrate. In these experiments acid disappearance plus lactate formation accounted for the pyruvate removal ( $-Q_{\text{keto}}$ ) during both periods. This is surprising since, if pyruvate be the first oxidation product of lactate, and if there be an equilibrium

Table IV. *Ox retina: arsenite and pyrophosphate*

Substrate	Glucose	$-Q_{\text{O}_2}^*$	R.Q.	$Q_{\text{A}}$	$Q_{\text{LA}}$	$Q_{\text{keto}}$
Lactate	—	12.6	0.94	-3.4	-3.6	+0.8
Lactate + arsenite	—	2.1	—	+2.0	-6.7	+4.0
Pyruvate	—	12.3	1.12	-2.6	+0.9	-6.0
Pyruvate + arsenite	—	0	—	+1.3	+1.7	+1.3
No addition	—	6.4	0.93	-1.0	-0.5	-0.3
Pyrophosphate	—	7.1	0.89	-0.3	+0.2	0
Lactate	—	11.1	0.85	-2.3	-4.3	+0.5
Lactate + pyrophosphate	—	16.2	0.95	-2.9	-2.5	-0.1
No addition	—	6.9 (21.9)	0.85	-0.2	-0.2	0
Pyrophosphate	—	5.2 (12.7)	1.06	-0.6	-0.3	0
No addition	+	9.8 (26.6)	0.86	+8.4	+6.3	+0.1
Pyrophosphate	+	15.2 (27.2)	0.98	+9.9	+11.8	+0.2
No addition	—	7.4 (13.6)	0.99	-0.9	—	—
Pyrophosphate	—	9.3 (12.9)	1.00	-0.8	—	—
Lactate	—	11.1 (15.8)	0.90	-2.3	—	—
Lactate + pyrophosphate	—	14.5 (20.1)	0.97	-3.1	—	—

\* See Table I.



between lactate and pyruvate, one would expect to find with added pyruvate the same apparent accumulation of acid in the first period and extra removal of acid in the second period, as was found with added lactate.

In the presence of  $M/1000$  arsenite, which inhibits the oxidation of keto-acids [Szent-Györgyi, 1930; Krebs & Johnson, 1937], the oxidation of pyruvate was completely inhibited. The greater part of added lactate which disappeared was oxidized to pyruvate, but a small part was transformed into some acid not accounted for by pyruvate, perhaps the same acid which appeared in other experiments with lactate as substrate. The fact that lactate removal was greater in the presence than in the absence of arsenite might be due to the inhibition of re-reduction of pyruvate to lactate.

The discrepancy between lactate and acid disappearance contrasted with the equivalent pyruvate and acid disappearance, and the results of the experiments with arsenite, suggest that lactate can be oxidized to some acid without being first converted into pyruvate, but it is difficult to picture what this process can be.

*Succinate* causes only a slight increase in  $O_2$  uptake and a lowering of the R.Q., without any appreciable acid disappearance, showing that if oxidized at all, it goes only as far as fumarate. Elliott & Greig [1937] report a very low succinioxidase content in ox retina.

*Fumarate and malate* form an equilibrium mixture in the presence of retina but they do not seem to be oxidized further. In the presence of fumarate there is always an unexplained lowering of the R.Q. which does not always occur with malate. This is manifest in a lowering of  $Q_{CO_2}$  and not in an increased  $Q_{O_2}$ .

*Oxaloacetate*. Retina accelerates the decarboxylation of oxaloacetate slightly. Aerobically the pyruvate formed is further oxidized; under anaerobic conditions it is reduced to lactate.

*Acetate* does not seem to be oxidized at all by retina. It may have some unexplained effect on the metabolism since in the presence of glucose it seemed to increase the  $Q_{O_2}$  somewhat and the R.Q. was lowered, but no increased acid disappearance occurred.

*Formate, oxalate and  $\beta$ -hydroxybutyrate* do not affect the metabolism of retina.

*Citrate and  $\alpha$ -ketoglutarate*, which are members of Krebs's cycle for the oxidation of carbohydrate in muscle, are not metabolized by intact retina, although Grönwall [1937] reports the presence of citric dehydrogenase in retina.

#### CHICK EMBRYO

Six-day chick embryos were used. Two embryos were placed in each vessel, and were snipped with scissors several times, inside the vessel, before adding the medium. Although in the aerobic experiments the  $O_2$  uptake of the chopped and intact embryos was approximately the same, in the anaerobic experiments glycolysis was considerably higher with chopped embryos. The latter finding indicates either that damage to the tissue stimulates glycolysis, or that rates of diffusion of solutes limit the apparent rate of glycolysis in the intact embryo. With chopped embryos the variation in  $Q_{O_2}$  between duplicate experiments was small (8%). Results were calculated on the final dry weights, i.e. the dry weight of the tissue obtained at the end of the experiment.

Added glucose has little, if any, effect on  $O_2$  uptake but it does cause an increase in acid formation. The aerobic glycolysis over the usual experimental period is small, as has already been shown by Warburg *et al.* [1924] and Needham *et al.* [1937, p. 1198], but during the first few minutes of incubation the rate of

lactic acid formation is considerable,  $Q_{LA}$  for the 20 min. equilibration in the presence of glucose being 18.2, 17.5, 17.6. In the absence of glucose,  $Q_{LA}$  for the same period is 11.6, 13.9, 12.5, 11.9, 9.0. When no substrate is present, the rate of lactic acid formation falls off rapidly and the lactate formed during the equilibration period from native substrate is metabolized giving a negative  $Q_{LA}$  for the experimental period. In the presence of glucose the rate of lactate formation, although less than the initial rate, is greater than its rate of oxidation throughout the experimental period, so that  $Q_A$  and  $Q_{LA}$  for the experimental period have positive values.

Some lactate is produced anaerobically in the absence of substrate ( $Q_{LA}^{\Sigma} = 2.5$ , 4.0 for the experimental period), presumably from some intracellular precursor.

Table V. *Chick embryo*

Substrate	Glucose	- $Q_{O_2}$	R.Q.	$Q_A$	$Q_{LA}^*$	$Q_{keto}$
No addition	+	11.5	0.99	+1.5	+2.0	+0.4†
No addition	.	10.9	1.08	+1.6	+2.3	+0.5†
No addition	-	11.9	0.95	-2.4	-1.4	0.2
No addition	-	12.3	0.90	-2.8	-1.6	0
Pyruvate	-	11.7	1.13	-3.2	+0.6	-3.4
Lactate	-	12.8	0.93	-3.3	-4.3	+0.2
No addition	-	12.1	0.95	-2.1	-0.9	-0.2
Succinate <i>M</i> /100	-	11.5	0.93	-1.9	-0.1	-0.2
Fumarate	-	12.0	0.79	-1.3	[-0.8]	0
Malate	-	12.4	0.97	-3.1	[-0.5]	0
Acetate	-	12.0	0.91	-2.5	-2.6	0
No addition	-	12.4	0.92	-1.9	—	—
Succinate <i>M</i> /100	-	12.2	0.93	-2.5	—	—
Succinate <i>M</i> /25	-	11.8	0.91	-2.2	—	—
Oxalate	-	12.5	1.01	-2.7	—	—
No addition	+	11.2	1.06	+1.1	-1.7	+0.3
Lactate	+	10.1	0.97	+0.9	-2.5	+0.3
Pyruvate	+	11.3	1.17	-1.2	+2.9	-3.6
Fumarate	+	10.2	1.00	+0.5	[1.2]	-0.4
No addition	-	12.5	0.83	-0.7	—	—
Citrate	-	10.7	0.78	-1.2	—	—
$\alpha$ -Ketoglutarate	-	10.5	0.87	-1.0	—	—
Oxalate	-	14.1	0.98	-3.0	—	—

*Anaerobic*

Substrate	Glucose	$Q_{CO_2}^{\dagger}$	$Q_A^{\dagger}$	$Q_{LA}$	$Q_{keto}$
No addition	+	+0.8	+6.5	+6.5	†
No addition	+	0	+10.5?	+6.9	†
No addition	+	0	+16.1	+13.3	—
No addition	+	+1.8	+17.3	+13.4	—
No addition	-	0	+2.9	+2.5	0
Pyruvate	-	0	+2.7	+3.8	-2.6
No addition	+	+0.6	+15.7	+13.8	0
Pyruvate	+	+0.9	+12.6	+13.4	-0.9
No addition	-	3.3?	+1.1?	+4.0	-0.1
		(+170 $\mu$ l. $CO_2$ )	(+55 $\mu$ l. $CO_2$ )		
Oxaloacetate	-	5.0	-3.3	+2.1	-0.3
		(+310 $\mu$ l. $CO_2$ )	(-204 $\mu$ l. $CO_2$ )		
(Oxaloacetate, no tissue)	-	(276 $\mu$ l. $CO_2$ )	(-310 $\mu$ l. $CO_2$ )		

\* Figures in brackets under  $Q_{LA}$  refer to estimations given wholly or partly by malate.

† Intact embryo.

‡ Figures for  $\mu$ l.  $CO_2$  given in parenthesis under  $Q_{CO_2}^{\dagger}$  represent the total respiratory  $CO_2$  evolution during the experimental period. The figures for  $\mu$ l.  $CO_2$  under  $Q_A^{\dagger}$  represent the total  $CO_2$  evolved or absorbed as the result of acid changes.

The rate of anaerobic glycolysis for the equilibration period is higher,  $Q_{LA}^N = +15$ ,  $+19$ , in the absence of substrate. Needham & Nowiński [1937, p. 1170], using smaller embryos, found a lower anaerobic autoglycolysis,  $Q_{LA}^N = 0.65$ .

In the presence of glucose, the initial rate of anaerobic glycolysis (for the first 20 min.) gives values of  $Q_{LA}^N = +32$ ,  $+35$ , but the rate falls off rapidly and much lower values are found during the usual experimental period after the time taken for equilibration.

In aerobic and anaerobic experiments, a small amount of pyruvate is usually found in the left-hand vessels after the equilibration period (c. 0.10 mg., corresponding to  $Q_{keto} +3$ ,  $+2$ ). In aerobic experiments, when no substrate is added,  $Q_{keto}$  for the experimental period is negative, showing that the pyruvate which accumulates quickly at first is then oxidized away faster than it is formed.

*Lactate.* As mentioned above, some lactic acid is rapidly formed during the first 20 min. equilibration period from metabolites already present in the cell, and during the experimental period part of this is oxidized. The lactate formed from native substrates seems to be almost sufficient, in most cases, to maintain lactate oxidation at its maximum rate during the experiment, since with added lactate the extra acid disappearance is usually slight and  $O_2$  uptake is not affected.

*Pyruvate.* Added pyruvate has no effect on  $O_2$  uptake, but that it is metabolized is evidenced by ketone and acid disappearance, as well as by the raised R.Q. The rate of pyruvate disappearance is about the same in the presence and absence of glucose. Some of the pyruvate is reduced to lactate. Anaerobically the only effect appears to be a reduction of some pyruvate to lactate.

*Succinate, fumarate, malate and oxaloacetate* are not appreciably attacked by embryo. Even fumarase activity seems to be lacking in the coarsely chopped embryo, and in this respect embryo differs from other tissues studied. Fumarate usually causes a lowering of the R.Q. as was also found with retina. Blazs6 [1936] found that rat embryonic tissue did not attack fumarate or oxaloacetate. He, like Elliott & Greig [1937], found succinoxidase activity in the rat embryo, but Elliott & Greig found only slight activity in chick embryo.

*Acetate, citrate and  $\alpha$ -ketoglutarate* are not metabolized.

*Oxalate* seems to be oxidized, as indicated by acid disappearance and increased  $O_2$  uptake. The Ringer-Krebs medium contains  $Ca^{++}$  which precipitates with added oxalate, but a considerable amount of oxalate still remains in solution.

## DISCUSSION

Compared with other tissues studied in this series of investigations retina most nearly resembles brain, but the aerobic glycolysis is higher and the succinoxidase content is lower than in brain. Retina oxidizes added lactate and pyruvate rapidly to  $CO_2$  and  $H_2O$ ; it oxidizes succinate to some extent but does not appreciably oxidize added fumarate, acetate,  $\alpha$ -ketoglutarate,  $\beta$ -hydroxybutyrate or citrate.

The oxidation of lactate seems to take place in two well-defined steps—in the first stage lactate is oxidized to some intermediate acid which accumulates; in the second stage lactate oxidation ceases and the unknown intermediate is metabolized. Further investigation of this intermediate by larger scale experiments is necessary. The fact that pyruvate oxidation does not seem to occur in two steps suggests that there may be some decided difference between the modes of metabolism of these two substrates in retina.

Embryo differs from most other tissues studied in that added glucose, lactate or pyruvate have little effect on respiration. Needham *et al.* [1937] also found

that embryo (4–6 day) has reserves of an unknown nature which permit of a long-continued aerobic respiration in the absence of substrate; but they concluded that this stored metabolite was not a lactic acid precursor since they found only a negligible amount of lactic acid formed when no substrate is present ( $Q_1$ ,<sup>1</sup> usually below 0.5, p. 1197). Contrary to this we find with 6-day embryos, a large initial autoglycolysis,  $Q_{LA}$  for the first 20 min. +15 and for the experimental period  $Q_{LA}=2.5$ . It is possible that the enzymes are kept nearly saturated with lactate and pyruvate, and therefore addition of these substrates does not affect respiration appreciably. Added succinate, fumarate and malate have no effect at all.

*Concerning the theories of Szent-Györgyi and Krebs*

In ox retina and chick embryo, as in liver, brain, testis and tumours [Elliott *et al.* 1935; 1937] the metabolism of lactate and pyruvate does not follow the cycle of reactions involving intermediate formation and oxidation of succinate and malate which was found to occur in kidney cortex slices [Elliott & Schroeder 1934; Elliott *et al.* 1935]. However, the negative results found with these tissues do not prove that the catalytic systems postulated by Szent-Györgyi [1937] or Krebs [Krebs & Johnson, 1937; Krebs & Eggleston, 1938] play no part in the respiratory mechanism. It is possible to assume that the necessary enzymes occur in all the types of cells studied. For instance, malic dehydrogenase occurs in liver [Green, 1936] although added malate affects the metabolism of liver slices very little [Elliott *et al.* 1937]. But the cells of the more or less intact sliced or slightly chopped tissue may already contain sufficient of the substrates oxaloacetate-malate and fumarate-succinate to carry all the respiration according to either theory. Adding more malate might increase the formation of oxaloacetate, but the reaction would cease very early owing to the inhibitory action of very low concentrations of oxaloacetate [Banga & Szent-Györgyi, 1936; Green, 1936], unless the oxaloacetate were rapidly removed by re-reduction by donator systems (Szent-Györgyi's scheme) or by a synthesis with some other molecule to give citrate (Krebs's scheme). Therefore the rate of respiration, if determined by either of these schemes, would not be greatly affected by the addition of malate. The respiration rate would depend on the rate of reductive or synthetic removal of oxaloacetate and on the activity of the malic and succinic dehydrogenases and the various carriers. Of course the picture is likely to be altered if the tissue is disintegrated and, as a result, the malate and fumarate present are diluted. Then, as shown by Gözsy & Szent-Györgyi [1934], Stare & Baumann [1936], Krebs & Eggleston [1938] and others, addition of fumarate is necessary to keep the enzymes sufficiently saturated to maintain the respiration rate.

The case of succinate addition is rather different. The oxidation product of succinate, namely fumarate, does not inhibit succinate oxidation except in high concentration [Das, 1937]. Therefore addition of succinate should cause an increase in the respiration of intact tissue unless there is only just enough succinoxidase<sup>1</sup> activity to maintain the normal respiration. In all the tissues studied in this series, with the exception of embryo and certain tumours, added succinate does increase the  $O_2$  uptake. (In fact the full increase was not observed by Elliott *et al.* [1937] with liver, brain and testis slices, owing to the rapid using up of the small amount of succinate added (3 ml.  $M/100$ ) and possibly the entrance of diffusion effects [see also Rosenthal, 1937].) But with chick embryo and the tumours studied, the succinoxidase activity is not in excess of the

<sup>1</sup> The term "succinoxidase" is used as a name for the complete succinate-oxidizing system consisting of succinic dehydrogenase, cytochrome and cytochrome oxidase.

amount necessary to carry the whole respiration; actually determinations on disintegrated tissue [Elliott & Greig, 1938] have shown that chick embryo and the tumours in question are definitely deficient in succinoxidase. In chick embryo and some tumours, therefore, the normal respiration does not depend mainly upon the Krebs or the Szent-Györgyi system. In retina particularly, and in testis [Elliott *et al.* 1937, and unpublished results with large amounts of succinate], the rate of  $O_2$  uptake is increased by adding excess succinate but not as much as it is increased by adding lactate or pyruvate. Therefore, while the respiration without added substrate may possibly go through the succinate system, the entire respiration of retina and testis in the presence of lactate or pyruvate cannot be carried by the succinoxidase system, and so neither of the two theories describes the whole respiratory system in these cases. In the other tissues studied in this series, namely kidney, liver and brain, the results provide no evidence for or against either scheme.

In the case of kidney cortex slices, actual oxidative removal of malate was found, and pyruvate removal appeared to follow a cycle of reactions involving succinate and malate as intermediate steps [Elliott & Schroeder, 1934; Elliott *et al.* 1935; Elliott & Greig, 1937]. In this case oxaloacetate formed by malate oxidation must be removed by a process which is neither reduction back to malate, nor synthesis with some molecule provided from outside the cycle, since malate, succinate etc., are removed and not regenerated. It also seems unlikely that spontaneous decarboxylation alone can account for the removal of oxaloacetate, as was first suggested. It is possible that, in kidney slices, the oxaloacetate is removed by a condensation with itself or with some other substance which is derived from it and not provided from outside the cycle. This problem is being studied. The actual transfers of hydrogen to oxygen concerned in the oxidative steps of the kidney cycle may take place via the Szent-Györgyi transportase systems.

#### SUMMARY

1. The metabolism by ox retina and chick embryo of lactate, pyruvate and various other compounds has been studied. Previous papers of the series indicated that in rat kidney cortex lactate is oxidized to pyruvate and that the pyruvate is removed largely by a cycle of reactions involving the successive formation of succinate, fumarate, malate, oxaloacetate and finally pyruvate in half the original amount. In neither of the tissues dealt with in the present investigation does this cycle of reactions occur.

2. In retina, lactate and pyruvate are oxidized quite rapidly. Succinate is oxidized slightly to fumarate and there is an equilibrium between fumarate and malate but no further oxidation. Fumarate causes an unexplained lowering of the R.Q. Acetate, formate,  $\beta$ -hydroxybutyrate, citrate and  $\alpha$ -ketoglutarate are not oxidized to any extent. Like liver, brain and testis, retina shows a much higher aerobic glycolysis rate during the first few minutes' equilibration than occurs for the experimental period. The intermediates in lactate and pyruvate oxidation have not been identified; manometric work shows that some acid intermediate other than pyruvate is formed from lactate during the first stage of the experiment, and later, when lactate is no longer oxidized, the unknown acid acts as substrate.

3. In embryo, added lactate and pyruvate are metabolized to some extent. Succinate, fumarate, malate,  $\alpha$ -ketoglutarate and citrate are not attacked. The coarsely chopped tissue shows no fumarase activity. Oxalate is oxidized.

4. The results in this series of papers are discussed in relation to the theories of Szent-Györgyi and Krebs.

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# LVII. THE ACTION OF LIGHT ON SUBSTANCES RELATED TO ERGOSTEROL

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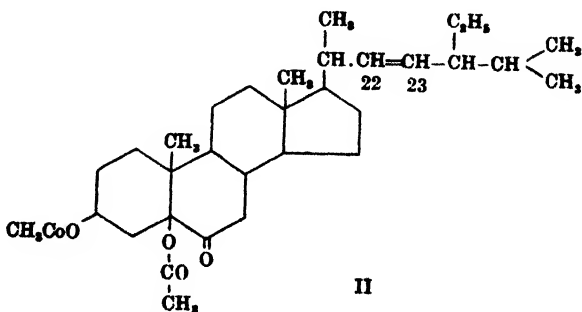
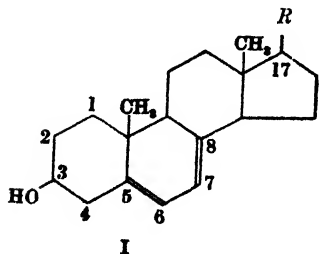
THE brilliant work of Windaus and his colleagues, and of other workers, on 7-dehydrocholesterol [Windaus *et al.* 1935; Windaus *et al.* 1936; Boer *et al.* 1936; Schenck, 1937; Windaus *et al.* 1937] has culminated in the isolation of the related crystalline vitamin D<sub>3</sub>, not only from irradiated 7-dehydrocholesterol itself, but also from the liver oil of the tunny [Brockmann, 1936; 1937; Brockmann & Busse, 1937], halibut [Brockmann, 1937], and cod [Zucker *et al.* 1938].

Of particular interest in connexion with this work is the question of the possible existence of other naturally occurring antirachitic substances, in addition to calciferol and vitamin D<sub>3</sub>. On biological grounds Bills *et al.* [1937; 1938] consider proven the presence of such compounds in fish liver oils. Further, Windaus & Trautmann [1937] have obtained a crystalline vitamin D<sub>4</sub> from irradiated 22-dihydroergosterol (Ia), while Wunderlich [1936] showed that his 7-dehydrostigmasterol (I b) possessed provitamin D activity. As Bacharach [1936] emphasized, it seemed remarkable, in view of these results, that the 7-dehydrostigmasterol (I c) of Linsert [1936] had no such properties.

Recently, the "cholanic acid analogue of ergosterol", 3-hydroxy- $\Delta^{5,7}$ -choladienic acid (I d) was prepared, with the use of an improved method for the introduction of the C<sub>7-8</sub> double bond [Haslewood, 1938]. This method has now been applied to the preparation of 7-dehydrostigmasterol. The final product was apparently identical with that of Linsert but certain of the intermediates showed different characteristics. In the preparation, also, 3:5-diacetoxy-6-keto- $\Delta_{22}$ -sitostene (II) was isolated.

On treatment with sunlight and eosin, in absence of air, the acid (I d) and 7-dehydrostigmasterol both gave crystalline insoluble products, but insufficient material was available for their complete characterization.

- I (a)  $R = -\text{CH}(\text{CH}_3) \cdot \overset{22}{\text{CH}_2} \cdot \overset{23}{\text{CH}_2} \cdot \text{CH}(\text{CH}_3) \cdot \text{CH}(\text{CH}_3)_2$ .  
 I (b)  $R = -\text{CH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{C}_2\text{H}_5) \cdot \text{CH}(\text{CH}_3)_2$ .  
 I (c)  $R = -\text{CH}(\text{CH}_3) \cdot \text{CH}=\text{CH} \cdot \text{CH}(\text{C}_2\text{H}_5) \cdot \text{CH}(\text{CH}_3)_2$ .  
 I (d)  $R = -\text{CH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$ .  
 I (e)  $R = -\text{OH}$ .



With the generous co-operation of Mr A. L. Bacharach, Dr E. L. Smith and Dr F. A. Robinson of the Glaxo Laboratories, the hydroxycholadienic acid and its ammonium salt, and also 7-dehydrostigmasterol have been irradiated and the products tested on rats for antirachitic activity. The tests, in the Glaxo Laboratories, and in those of the author, showed that the provitamin activity of the acid and its salt was not more than 1/125–1/400 of that of ergosterol; while irradiated 7-dehydrostigmasterol also had not more than 1/125–1/400 of the antirachitic activity of ergosterol similarly treated.

Recently, Dimroth & Paland [1939] have similarly found negative results in the case of the 3:17-dihydroxyandrostadiene (1*e*) of Butenandt *et al.* [1938]. Compounds of this group seem to show great specificity in the possession of provitamin properties.

#### EXPERIMENTAL

Analyses were microanalyses by Dr A. Schoeller. Melting points are uncorrected. Ethereal solutions were dried with anhydrous sodium sulphate.

*7-Dehydrostigmasterol.* Stigmasterol acetate (10 g., m.p. 142–143°) in acetic acid (500 ml.) was oxidized for a total time of 4 hr. at 50–55° with a solution of chromic anhydride (8 g. CrO<sub>3</sub> in 20 ml. water) and acetic acid (80 ml.); added slowly with continuous stirring during 70 min. After dilution to about 3 l. with water, the product was allowed to stand overnight and the precipitate collected and dissolved in ether. The ether was washed with NaOH and water, dried and evaporated. The residue, from ethyl alcohol, yielded white leaflets of 7-ketostigmasterol acetate, m.p. 184–185° (Linsert: m.p. 183°). The mother liquors were diluted in three stages with water, and filtered twice from crystalline precipitates. Finally, needles were deposited, and these were collected and purified by recrystallization from methyl alcohol and methyl alcohol-light petroleum (twice). In this way, 3:5-diacetoxy-6-keto- $\Delta_{23}$ -sitostene (II) was obtained as colourless needles, m.p. 189–190°. (Found: C, 74.8; H, 9.9%. C<sub>33</sub>H<sub>52</sub>O<sub>5</sub> requires C, 75.0; H, 9.9%.) 7-Ketostigmasterol acetate (1.5 g.) was reduced with aluminium isopropoxide, purified and the product (1.1 g.) converted into the dibenzoate exactly as described for 7-keto-3-acetoxy- $\Delta_5$ -cholenic acid [Haslewood, 1938]. The dibenzoate of 7-hydroxystigmasterol crystallized from methyl alcohol-acetone in white needles, m.p. 184–186° (Linsert: m.p. 156–158°). (Found: C, 80.9; H, 8.8%. C<sub>43</sub>H<sub>56</sub>O<sub>4</sub> requires C, 81.1; H, 8.9%.) This product (0.7 g.) was refluxed for 8 hr. with redistilled dimethylaniline (5 ml.; b.p. 191–192°). The diluted product was acidified with HCl and ether-extracted. Evaporation of the washed and dried ether and crystallization of the residue from ethyl alcohol-benzene yielded white leaflets (0.3 g.). These were refluxed for 15 min. with alcohol (50 ml.), acetone (20 ml.) and 2*N* NaOH (2 ml.). The diluted mixture was ether-extracted, and the residue from evaporation of the washed and dried ether was crystallized from alcohol until the product (70 mg.) formed colourless needles of constant m.p. 149–151° (Linsert: m.p. 154°). 7-Dehydrostigmasterol gave the colour reactions of ergosterol. The acetate (prepared in the usual way with acetic anhydride and pyridine at 100° for 15 min.) crystallized from alcohol-benzene in white leaflets, m.p. 169–171° (Linsert: m.p. 172°).

*Effect of sunlight and eosin.* (a) On 7-dehydrostigmasterol: this substance (15 mg.) with eosin (15 mg.) was gently boiled in a 5 ml. flask with ethyl alcohol (4 ml.) and benzene (1 ml.). While still hot, the flask was tightly stoppered, and was then allowed to stand for some weeks in a sunny place. The precipitated needles were collected, and recrystallized from benzene-alcohol. White crystals m.p. 203–204° were obtained.



(b) On 3-hydroxy- $\Delta^{5,7}$ -choladienic acid: this acid (20 mg.), eosin (20 mg.) and ethyl alcohol (5 ml.), were treated as above in a 5 ml. flask. The product consisted of white needles, M.P. 238–239°.

*Tests of provitamin activity.* (a) In Glaxo Laboratories: 5 mg. of the compounds tested were irradiated in a quartz tube placed about 4 cm. from a mercury vapour lamp. The solutions were rapidly stirred, and a current of  $N_2$  passed through them during the irradiation. The resulting solutions (except in the case of the ammonium salt of the acid (I), which was fed directly to rats) were evaporated, and the residues dissolved in olive oil and fed to rachitic rats. The technique of the assays was that of the "line" test. The following results were obtained:

Substance tested	Irradiation time (min.)	Activity of product
Hydroxycholadienic acid as the ammonium salt, in water (50 ml.)	2	Not more than 10 I.U. per mg.
The above acid in ethyl alcohol (25 ml.)	2, 5, 15 and 45	Not more than 10 I.U. per mg.
7-dehydrostigmasterol in ethyl alcohol (25 ml.)	3	Not more than 10 I.U. per mg.
Ergosterol in alcohol (25 ml.)	3	Not more than 4000 I.U. per mg.

(b) In the author's laboratory: the mercury vapour lamp was 5.5 cm. from the quartz tube. The residues from evaporation of the solutions were dissolved in olive oil and tested on Wistar rats. The standard (10 I.U.) was an olive oil solution of pure calciferol. The "line" test and X-ray technique were used in the assays. The following results were obtained:

Substance tested	Irradiation time (min.)	Activity of product
Hydroxycholadienic acid (1 mg.) in ethyl alcohol (5 ml.)	10	<25 I.U. per mg.
7-Dehydrostigmasterol (1 mg.) in ether (5 ml.)	10	<25 I.U. per mg.
0.6 mg. of the above in ether (3 ml.)	10	<25 I.U. per mg.

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# LVIII. BLOOD AMMONIA<sup>1</sup>

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## I. INTRODUCTION

CONCERNING the normal content of free  $\text{NH}_3$  in human venous blood, it was previously shown [Conway, 1935] that the characteristic value of this was either zero or below the analytical level (less than 1 part in 30 millions) but that when blood was shed into an open vessel a rapid rise in the free  $\text{NH}_3$  content occurred in the first 5 min. after shedding.<sup>2</sup> Observations of the same kind are described in the present paper on the rabbit (ether anaesthesia) and the fowl.

The formation and origin of  $\text{NH}_3$  after shedding is not only of interest in relation to deamination in the blood stream itself, but also by comparison with similar processes in tissues. This blood  $\text{NH}_3$  is here described under three headings, namely, the "alpha"  $\text{NH}_3$  which originates immediately after shedding and for which some evidence is given that it may arise from adenosine (or possibly from minute concentrations of free adenylic acid), the "beta"  $\text{NH}_3$  which is shown to derive after a series of stages from adenylypyrophosphoric acid, and the "gamma"  $\text{NH}_3$  which appears to come either from vegetable adenylic acid or from adenyldoxyribonucleotide or alternatively from substances liberating these in plasma and in the red corpuscles. The "beta" and "gamma" moieties correspond to the B and A fractions of Heller & Klisiecki [1932].

From the evidence presented it would appear that most if not all the blood  $\text{NH}_3$  forming in sterile blood collected by open shedding derives finally from adenosine.<sup>3</sup> This is not necessarily so for the fowl's blood which can also deaminate guanosine, cytidine and even adenine freely. Neither does it hold for mammalian blood maintained at pH 7.4 by suitable  $\text{CO}_2$  tension. The "beta"  $\text{NH}_3$  then derives very largely from adenylic acid, only part of the  $\text{NH}_3$  appearing via adenosine.

Concerning the specificity of the deamination in blood a large number of substances containing amino (or volatile amine) groups has been studied for possible deamination in blood, but no other substances besides adenosine or associated compounds have been found to yield  $\text{NH}_3$  (or amines) at appreciable rates or at rates comparable with the normal formation of ammonia in shed blood. Thus we have repeatedly found that arginine, if added in small amounts to shed rabbit's blood or buffered after addition at a pH between 7 and 8, only gives negative results. It may be noted, however, that the main point in view was to ascertain the origins of the blood  $\text{NH}_3$  and that the conditions of the investigation did not differ very widely from the normal.

<sup>1</sup> A preliminary report of this work appeared in *Nature*, 139, 1937, and 142, 1938.

<sup>2</sup> Results on the normal dog similar to these findings for the human subject have been reported to us by H. Kropowski, the Pathological Laboratory, Warsaw University.

<sup>3</sup> The presence of adenosine deaminase in plasma was shown by one of us [Cooke, 1936] prior to Drury's communication to the Physiological Society.

II. THE NORMAL CONTENT OF  $\text{NH}_3$  IN CIRCULATING BLOOD*Methods*

*Experimental.* The blood either from an arm vein in the resting human subject, or from the carotid in the anaesthetized rabbit (ether anaesthesia) or from the severed neck vessels in the fowl has been investigated. The method of collecting the blood has been already described [Conway, 1935] the same procedure being adopted, using a carotid cannula in the rabbit.

*Revised method.*

*Analytical.* The method of suspended absorption [Conway, 1935] with the special micro-diffusion "unit" [Conway & Byrne, 1933] has been used, but revised in certain points. The essential differences of the revised method consist first in standardizing the whole procedure with each series of determinations by means of a duplicate control on standard ammonium sulphate solutions. When this is done with the same glassware, all corrections for this and the alkali as well as temperature effects on the absorption rate are eliminated. Also it is recognized that the emission rate of  $\text{NH}_3$  from a blood-carbonate mixture is not quite the same as from a water-carbonate mixture. The previous conclusion in this respect [Conway, 1935] was based on a few recoveries under conditions not quite ideal for the comparison, though it was pointed out that a small difference was unimportant. A very large number of such recoveries of comparatively high  $\text{NH}_3$  quantities (to avoid appreciable fluctuations of the blood  $\text{NH}_3$  throughout the procedure) has since been carried out.

The  $\text{NH}_3$  formed by the alkali in addition to the preformed  $\text{NH}_3$  in blood has also been carefully studied. It has been found to be strictly linear with the time over a few hours. No essential difference from the previous conclusions has been found, but a temperature factor has been included which, however, for the short absorption periods is scarcely significant. In Table I are incorporated the necessary details for the revised method. The allowances for the alkali action on the blood have been calculated with the help of the formula previously given [Conway, 1935] and correspond with the experimental findings.

*Calculation of the blood ammonia using the revised method.*

This is shown by an example.

*Example 1.* (All titrations carried out in duplicate, the control solution containing  $2\text{ }\mu\text{g./ml. NH}_3\text{-N.}$ )

Titration of "blank" unit	...	35.6 large divisions
Titration of "analytical" unit	...	31.3     "
Titration of "control" unit	...	14.2     "
Time of absorption	...     ...	10 min.
Room temperature	...     ...	15°

The amount of absorption in the analytical unit corresponds to  $35.6 - 31.3$  or to  $4.3$ , and in the control to  $21.4$  large divisions (each large division of the burette [Conway, 1934] corresponds to  $0.01$  ml. of  $0.0004\text{--}0.0005\text{N Ba(OH)}_2$ ).

$$\text{Calculation: } \frac{4.3}{21.4} \times 1.15 \times 2.00 = 0.462\text{ }\mu\text{g./ml. NH}_3\text{-N.}$$

The factor  $1.15$  derives from Table I and  $2.00$  represents the concentration in the control.

From the gross value of  $0.462$ ,  $0.04$  is subtracted to allow for the special de-aminating action of the carbonate as given in Table I, column 4.

Final result =  $0.42 \mu\text{g./ml. NH}_3\text{-N}$ . Before titrating a series with  $\text{Ba(OH)}_2$  it is our custom to run out about 7–10 ml. from the burette, and also to have the cork of the reservoir bottle and of the soda-lime guard painted and sealed with melted paraffin. It will be seen that with the revised method changes in  $\text{Ba(OH)}_2$  strength are of no consequence, but it is advisable at the same time to change the solution about once weekly. Table II summarizes a number of "recoveries" of  $\text{NH}_3$  from blood, with the error range.

Table I

Absorption time min.	Ratio of $\text{NH}_3$ absorbed from water-carbonate to that from blood-carbonate mixture	The absorption as an approximate percentage of the total $\text{NH}_3$ in the aqueous solution %	Allowance in the final result for the special deammonating action of the alkali at different temperatures, $\mu\text{g./100 ml. NH}_3\text{-N}$				
			25°	20°	15°	10°	0°
10	1.15	52	11	7	4	3	1
12	1.14	57	14	8	5	3	1
14	1.12	61.5	16	10	6	4	2
16	1.11	65.5	19	12	7	5	2
18	1.11	69	22	14	9	5	2
20	1.11	72	25	16	10	6	2

In the above table (made out for the pyrex "unit" as made by Messrs A. Gallenkamp and Co., London) it is understood that 1 ml. blood or water is added to 1 ml. of saturated  $\text{K}_2\text{CO}_3$  in the outer chamber from pipettes exactly calibrated for this purpose, and that the mixing is carried out with a fixed routine of 15 rotations in approximately 15 sec. The allowances in the fourth column for the special deammonating action of the alkali have a temperature coefficient of 10 % per degree.

Table II. "Recoveries" of  $\text{NH}_3$  by the method of suspended absorption (revised)

Fluid examined	$\text{NH}_3$ added per ml. fluid $\mu\text{g. N}$	$\text{NH}_3$ recovered. Mean value $\mu\text{g. N}$	No. of observations	Range $\mu\text{g. N}$
Human blood	91.6	91.8	11	93.6 – 88.8
Rabbit plasma	91.6	91.6	6	94.6 – 87.1
Water	91.6	91.0	20	95.3 – 87.1
Human blood	1.92	1.91	1	—
Human blood	0.98	0.96	5	1.05 – 0.88
Human blood	0.39	0.36	2	—

The blood used in the above "recoveries" was freshly shed into an open flask. Usually 10–20 ml. samples were taken, 0.2 ml. of an  $\text{NH}_3$  solution in saline being added and a similar amount of saline without  $\text{NH}_3$  to a control sample.

For the first five groups 1 ml. of  $N/200$  acid with indicator was added to the inner chamber and titrated after the absorption with  $N/150$   $\text{Ba(OH)}_2$ , and for the last three groups the customary solutions for the normal blood  $\text{NH}_3$  were used (namely,  $N/5000$  acid and  $N/2000$  alkali).

Of the 37 results in the first five groups none exceed 4.5 % deviation from the mean value. The coefficient of variation for the group is 2.4 %.

For the most exact procedure possible with the method and the conditions described, the "units" after being filled and ready for the determination, are left aside on the bench for  $\frac{1}{4}$  hr., the acid in the central chamber being titrated to the end-point, the fluid removed and replaced by a refill of acid and the lid replaced, after which the determination is carried out. This eliminates any small defect in cleaning and also traces of  $\text{NH}_3$  in carbonate and fixative, and with a little experience will be found to give surprisingly good and uniform results.

*The method of suspended absorption with plasma and other fluids.*

The process of suspending the absorption after a short time makes the micro-diffusion method one of great flexibility in the rapid collection of numerous data, and is advisable when, generally speaking, an error range of 2-5 % for the single determination is not considered too high (the suspension being made after 10-20 min.).

With plasma, the  $\text{NH}_3$  comes over at the same rate from a plasma-carbonate mixture as from a water-carbonate mixture so that the factors in column 2 of Table I are not used. The corrections for the extra ammonia in the last column of this table still apply and should be subtracted from the final result.

For all diluted blood and tissue extracts of the order of one in five or more, the calculation is simply the ratio of the  $\text{NH}_3$  absorbed to that from the control multiplied by the strength of the control. The absorbed  $\text{NH}_3$  is represented as alkali divisions on the burette.

*Note on the cleaning of the "units".* The following procedure has been found suitable for all purposes. After rinsing with cold and hot water, soap and a test tube brush are used, then the "units" are well rinsed and soaked in dilute acid containing indicator (about  $N/200 \text{ H}_2\text{SO}_4$ ) for 15 min. (the "units" being filled to the top). After repeated rinsing with distilled water they are dried in air.

### Results

*The normal resting content of  $\text{NH}_3$  in human blood.*

Using the revised method the previous conclusions [Conway, 1935] were confirmed. The  $\text{NH}_3$  content of venous blood from the resting human subject on shedding into an open flask rises swiftly as if from zero to reach a mean value of  $0.41 \mu\text{g./ml. NH}_3\text{-N}$  within 5 min., after which it rises at about the rate of  $0.006 \mu\text{g./ml. NH}_3\text{-N/min.}$  at  $20^\circ$ . The curve of the median values of successive groups of 11 observations ranged in order of time is given in Fig. 1 (upper curve). The statistical description of variability remains as before [Conway, 1935]. For the time interval of 3-10 min. after open shedding, within which the mean value alters very little, the range found in 28 observations on 12 human subjects was from  $0.21$  to  $0.58 \mu\text{g./ml. NH}_3\text{-N}$ , the range being  $0.36$ - $0.58$  when observations on two subjects at the unusually low room temperature of  $12^\circ$  are excluded. Fig. 2 shows the course of the formation in pine subjects for whom a rather extended series was taken. The two lowest of these curves were taken at a room temperature of  $12^\circ$ .

*$\text{NH}_3$  content of rabbit's blood (short ether anaesthesia).*

Like the human venous blood there is a rise immediately after shedding into an open vessel. This "alpha" rise begins likewise from a concentration apparently zero—or at least not greater than 1 part in 10 million. The curve of the median values of successive groups of 11 observations on 9 rabbits is given in Fig. 1 (lower curve). (The median values are chosen in order to give the most characteristic curve.) The rabbit curve differs from the human in not reaching an apparent level after the 3rd to the 5th min. and, as will be seen in the next section, a much greater rise in the curve begins between the 10th and 20th min. Values ranging from  $0.24$  to  $0.59 \mu\text{g./ml. NH}_3\text{-N}$  were obtained from the 3rd to the 10th min. (32 observations). Fig. 3 shows graphically the variability and course of the formation for 7 rabbits within the first short period after shedding.

As shown below, the first rapid or "alpha" rise is eliminated by receiving the blood into an atmosphere containing  $\text{CO}_2$ .

*The  $\text{NH}_3$  content of fowl's blood after open shedding.*

Three experiments on the fowl's blood showed a similar but much more rapid rise than for the rabbit or human subject. The blood  $\text{NH}_3$  appears to rise here also from the zero position.

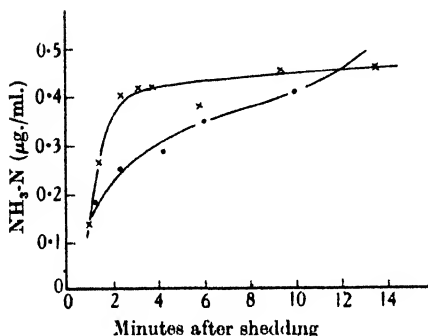


Fig. 1.

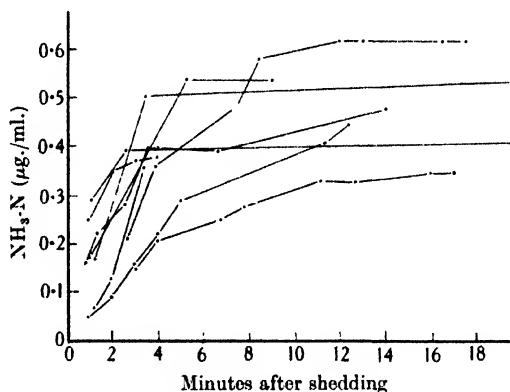


Fig. 2.

Fig. 1. Characteristic curves of the blood ammonia for man (crosses) and rabbit (dots) after shedding into an open flask. Room temperature. Each point is the median of consecutive samples of eleven observations ranged in order of time after shedding.

Fig. 2. Serial curves for nine human subjects, mostly mean of duplicates.

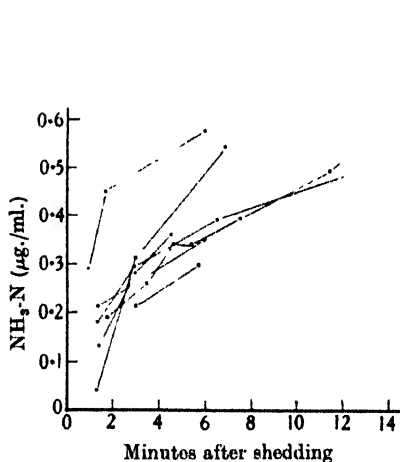


Fig. 3. Serial curves on 7 rabbits, mostly triplicate determinations.

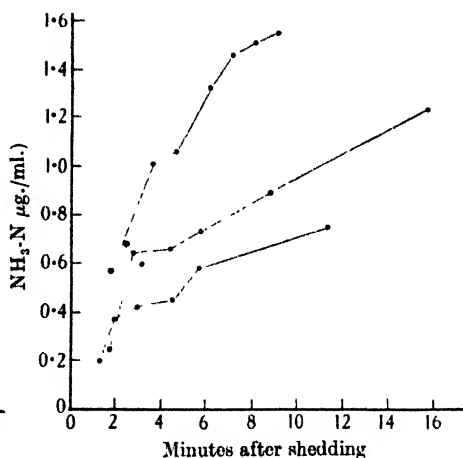


Fig. 4. Serial curves on 3 fowls, means of duplicates.

### III. THE $\text{NH}_3$ FORMING IN SHED STERILE BLOOD OF MAN AND RABBIT—ITS STAGES AND ORIGINS

**Total  $\text{NH}_3$ .** The total  $\text{NH}_3$  forming in the blood of man and rabbit is shown in Table III. The blood over the long periods was sterilized with thymol or toluene, being stored in vaccine bottles with the usual vaccine technique of capping etc.

It will be seen from Table III that total amounts of approximately  $13 \mu\text{g./ml.}$   $\text{NH}_3\text{-N}$  develop in human blood over several days at room temperature and about 20 in rabbit blood.

Table III

Subject	Blood examined	$\mu\text{g./ml. NH}_3\text{-N}$ formed after shedding					
		4 hr.	6 hr.	24 hr.	48 hr.	72 hr.	96 hr.
Rabbit	Whole blood	11.1 (3)	18.8 (3)	18.2 (7)	20.3 (3)	—	24.3 (2)
	Plasma	—	—	3.6 (5)	5.4 (2)	—	—
	Plasma (buffered with borate)	—	—	2.7 (2)	4.0 (2)	—	—
Man	Whole blood	—	—	7.4 (6)	13.1 (2)	13.8 (2)	—
	Plasma	0.7 (2)	—	1.5 (2)	—	—	2.7 (2)
	Plasma (borate buffer)	—	—	1.4 (3)	1.3 (3)	1.3 (1)	—

The numbers in brackets give the totals of individual subjects. All experiments were conducted at room temperature (mean of  $18^\circ$ ).

The figure for the whole blood  $\text{NH}_3$  in human and rabbit bloods after 24 hr. and longer periods agree with those of Parnas [1925] and of Stanojevic [1938] for the human subject. Earlier figures for the  $\text{NH}_3$  in human blood after 24 hr. given by one of us [Conway, 1935] were too low owing to undue exposure of the blood, which then during this time loses  $\text{NH}_3$  very appreciably, this exposure being carried out in connexion with a study of the effect of  $\text{CO}_2$  loss. All vessels containing blood collected by open shedding throughout the present study were stoppered after about 20 min. initial exposure.

In human plasma 1.3–2.7  $\mu\text{g./ml.}$  develop over 4 days with a mean of 1.4  $\mu\text{g./ml.}$   $\text{NH}_3\text{-N}$ . That there is practically no change from 1.4 over 3 days indicates that this may be taken as representing the total amount. The formation in rabbit plasma is greater than this. After 24 hr. about 3  $\mu\text{g./ml.}$   $\text{NH}_3\text{-N}$  have developed at room temperature. The formation has not then ceased but the further increase in a subsequent 24 hr. period does not exceed 1–2  $\mu\text{g./ml.}$

#### The “alpha” $\text{NH}_3$

This formation is almost complete in the first few minutes after open shedding as shown in Fig. 1.

It amounts to a mean of about 0.4  $\mu\text{g./ml. N}$  for rabbit and man. It does not appear on receiving the blood into  $\text{CO}_2$  as already shown for the human subject [Conway, 1935] and here as in Fig. 5 for rabbit blood.

It was previously demonstrated that  $\text{CO}_2$  in itself does not produce an apparent absence of  $\text{NH}_3$ . This has been repeatedly confirmed both for  $\text{NH}_3$  already formed in the blood and for recoveries of minute amounts of added  $\text{NH}_3$ . When, however, the blood  $\text{NH}_3$  is raised to the high level of 100  $\mu\text{g./ml. N}$  a somewhat reduced recovery—85 %—was found with the revised method allowing the usual 10 min. absorption period. In repeated experiments no such reduced recovery was found at the 1–10  $\mu\text{g./ml.}$  level, even with one atmosphere of  $\text{CO}_2$ ; and even if we were to allow this 15 % it would mean a recovery of 0.34  $\mu\text{g./ml. N}$  when 0.40 was really present. Such a difference is not significant for the present discussion.

The sudden lowering of the  $\text{CO}_2$  tension in open shedding can act either by raising the  $\text{pH}$  of the blood or in some specific manner. We have maintained the  $\text{pH}$  at 7.2 by reception into a maleic acid or a phosphate buffer solution forming about one-tenth of the whole blood collected, and with results shown in Fig. 6 (curve *B*). It is obvious that this procedure if anything only increases the initial rate of the formation compared with unbuffered blood (curve *A*) so that we must conclude that  $\text{CO}_2$  acts specifically.

It has been shown also [Conway, 1935] that carbamic acid itself is not the substance involved, but that some derivative containing an amino group is liberated, the amino group being then removed by ferment action. Adenosine could possibly fill this role, and the following is some evidence in support of such a view.

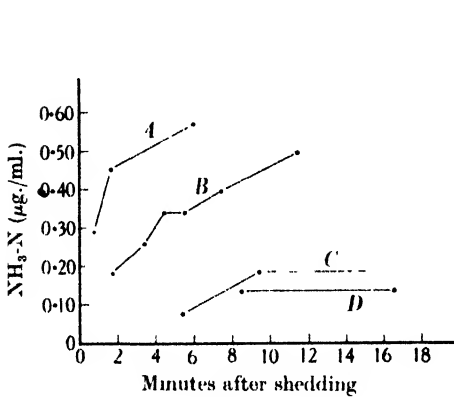


Fig. 5.

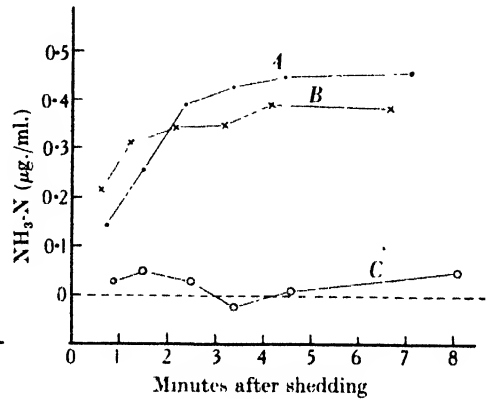


Fig. 6.

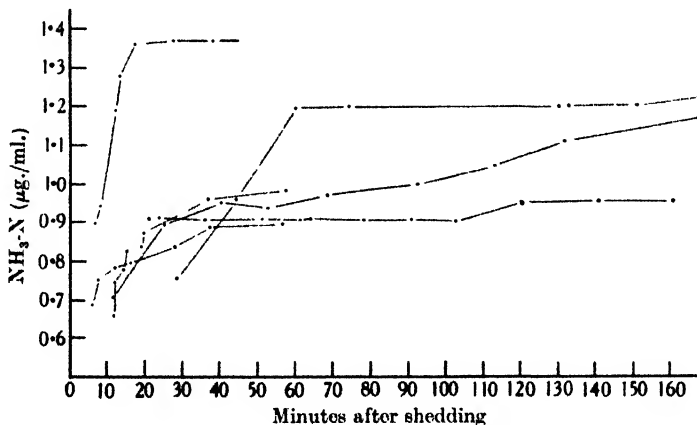
Fig. 5. Blood  $\text{NH}_3$ , 2 rabbits. A and B, open shedding; C and D, shedding into  $\text{CO}_2$  (100 mm.).

Fig. 6. Blood  $\text{NH}_3$ , 2 human subjects. A, open shedding, unbuffered; B, open shedding, buffered (7.2 pH); C, shedding into 100 mm. tension of  $\text{CO}_2$ .

*Evidence that adenosine is the amino substance involved in the "alpha" stage.*

(1) Out of 51 different substances investigated of physiological importance and containing amino or volatile amine groups, only adenosine, or substances that can liberate adenosine by dephosphorylation are deaminated in mammalian blood after shedding.

(2) From evidence given subsequently small quantities of adenosine must be continually liberated in plasma.

Fig. 7. Plasma  $\text{NH}_3$  formation in 6 rabbits. Room temperature.

(3) The differences in rate of formation of the "alpha" stage in plasma and in whole blood fits in well with the adenosine hypothesis. Small quantities of



adenosine added to whole blood are deaminated in about 1 min. or less, but take upwards of 30 min. in plasma. If the "alpha" rise occurs also in plasma it cannot be faster than this adenosine deamination if adenosine is involved.

Figs. 7 and 8 show the "alpha" rises in rabbit and in human plasma, and as will be seen both take about 30 min. to reach completion.

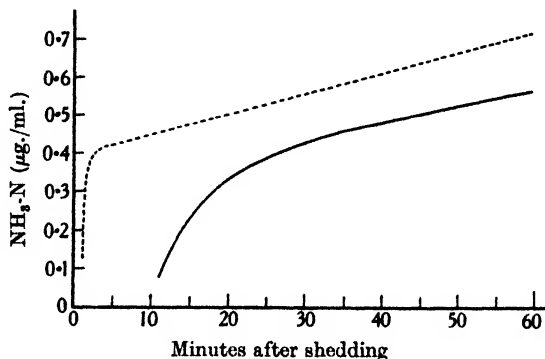


Fig. 8. Plasma  $\text{NH}_3$  formation for 3 human subjects (mean curve) after collecting into  $\text{CO}_2$  (100 mm. tension) with subsequent release of the gas by exposure in large flask. The dotted curve shows the  $\text{NH}_3$  formation in human whole blood.

The rabbit plasma was obtained by centrifuging immediately after open shedding; the human by centrifuging under 100–200 mm.  $\text{CO}_2$  tension and subsequent removal of the  $\text{CO}_2$  by shaking a small volume in a large conical flask.

(4) The pH effect on the  $\text{NH}_3$  formation in the "alpha" stage has a general correspondence with the effect on the deamination rate of adenosine. The "alpha" rise is inappreciable at 6.0 and 8.0 pH and seems most marked around a pH of 7.0. This question is again considered in the discussion at the end.

#### *The "beta" $\text{NH}_3$ and its phases*

The  $\text{NH}_3$  deriving from the breakdown of adenylypyrophosphoric acid is here termed the "beta"  $\text{NH}_3$ . The  $\text{NH}_3$  forming in whole blood after the "alpha" stage is a mixture of this  $\text{NH}_3$  and that deriving from another source which is present alone in plasma. This latter is termed the "gamma"  $\text{NH}_3$  and is subsequently considered.

#### *The relation of the A.T.P. content to the $\text{NH}_3$ formation*

##### *Methods.*

(a) *Ba salt determination.* The A.T.P. content was determined by a modification of the method of Parnas & Lutwak-Mann [1935] specially adapted for the absorption unit as used here. In this method the Ba salt is precipitated from a trichloroacetic filtrate and subsequently determined by the action of a muscle extract. This method allows also of a simultaneous determination of adenylic acid in the filtrate after the Ba precipitation but no appreciable quantity was found.

(b) *Determination of the total adenylypyrophosphate, adenylyl diphosphate and muscle adenylic acid.* This is carried out simply by the action of a muscle extract on the laked blood. At first strong extracts were used (1 in 3 to 1 in 5) and the  $\text{NH}_3$  largely removed after the addition of sodium carbonate etc. Much weaker extracts, however, may be used directly as was subsequently found (about 1 in

50 of frog or rabbit's skeletal muscle). The extract and laked blood were both buffered with maleic acid buffer ( $pH$  of 7.0,  $M/20$  in mixture) and mixed in equal volumes for the extract action, suitable controls being set up with extract and blood diluted with similar volumes of water. The total adenylyl  $NH_3$  is formed very quickly after mixing and is complete after about 15 min. at room temperature. The formation was in all cases studied from the time concentration curves.

*Results and blood  $NH_3$  comparisons.*

The determination of adenylypyrophosphoric acid in whole blood by the Ba salt method gave for 3 rabbits a mean of  $9.0 \mu g./ml.$  N and 9.5 with the direct action of the muscle extract. With the Ba salt method no appreciable amount of free adenylic acid was found, and this is in obvious agreement with the results of the second method, which therefore constitutes *an easy and accurate procedure for determining the A.T.P. in blood, and particularly suitable for numerous determinations.*

In 4 hr. at room temperature the total adenylyl (muscle) complex has just disappeared as such from rabbit blood and  $NH_3$  has formed in a practically equivalent relation as shown in Table IV. With human blood it takes about 24 hr.

Table IV

Subject	Total A.T.P. plus adenylic acid, $\mu g./ml.$ amino N	Change in concentration of adenyl complex $\mu g./ml.$ amino-N	Change in $NH_3$ $\mu g./ml.$ N	Time after shedding min.
Rabbit	8.4	-5.4	+7.3	180
	9.7	-9.7	+9.1	240
	10.3	-9.8	+10.3	240
Man	8.8	-5.7	+6.2	1440
	10.1	-5.7	+6.1	1440
	A.T.P. (from barium salt)	Change in A.T.P.		
Rabbit	7.8	—	—	—
	11.2	—	—	—
	8.1	-7.1	+(8.8)	225

The bracketed number in last line gives the mean  $NH_3$ -N formation in the 225 min. for many rabbits.

for a 60% disappearance of the A.T.P. and in this time there is an approximately quantitative appearance of free  $NH_3$ —a slight excess of  $NH_3$  being evident.

A study of the  $NH_3$  formation in the first 4 hr. in rabbit blood is therefore in effect a study of the "beta"  $NH_3$  over the complete range of its formation.

The figures for the total A.T.P. etc. in the two samples of human blood correspond but are somewhat lower than the data assembled by Buell [1935] for the total adenine nucleotide in human blood, passing through the uranium salt, hydrolysis of the nucleotide, removal of the uranium and determination of the free adenine nephelometrically after precipitating the silver salt. She obtained a range of 9.6–14.8 in numerous observations whereas the two samples here gave 8.8 and 10.1. Her determinations would have included both the adenosine-3-phosphoric and the adenosine-5-phosphoric acid groupings, whereas here only the adenosine-5-phosphoric acid grouping (muscle adenylic acid) would be determined.

*Stages in the breakdown of adenylypyrophosphoric acid leading to the formation of  $\text{NH}_3$*

(a) *Carbon dioxide is allowed to escape initially.* It has been recently shown by Kiessling & Meyerhof [1938] that in muscle A.T.P. is not present as such but rather as a dinucleotide yielding A.T.P. and behaving similarly to this in all the reactions hitherto observed. However this may be for the red corpuscle, we shall consider A.T.P. here as the starting point. Seeing that adenyldiphosphate is now considered to be a stage in the breakdown of A.T.P. in muscle [Lohmann, 1935] and that no free pyrophosphoric acid is formed, we may suppose that the A.T.P. is broken down in an analogous fashion in red corpuscles and that the diphosphate is a stage leading to adenylic acid. That this is not the final stage before deamination in shed blood after escape of  $\text{CO}_2$  is shown by the following evidence. The deaminase of adenylic acid is known to act very specifically [Schmidt, 1928] and does not act for example on yeast or vegetable adenylic acid. If we compare the actions of laked and diluted blood containing added bicarbonate ( $N/10$  in mixture) on solutions of the two nucleotides made up at a pH of 7.0 we obtain the results shown in Table V. When the nucleotide concentration is very high—1%—the

Table V

Added nucleotide in the laked blood mixture %	pH	NH <sub>3</sub> formation $\mu\text{g. N/ml./min.}$ (multiplied by the blood dilution)		Ratio
		Muscle adenylic acid	Yeast adenylic acid	
Rabbit A. Bicarbonate-nucleotide buffering. Blood dilution, 1 in 8.8; 0.05 <i>N</i> bicarbonate in the mixture; neutral nucleotide mixture added to laked blood				
1.0	7.5	12.2	0.890	0.07
0.5	7.7	5.45	0.238	0.04
0.2	7.9	1.32	0.132	0.10
0.1	8.0	0.037	0.032	0.85
0.05	8.2	0.0132	0.0123	0.93
Rabbit B. CO <sub>2</sub> -bicarbonate-nucleotide buffering. Blood dilution 1 in 5. 0.025 <i>N</i> bicarbonate in the mixture and CO <sub>2</sub> tension 38 mm.				
1.0	7.4	4.25	0.48	0.11
0.05	7.4	0.43	0.044	0.10

deamination of the muscle adenylic acid goes very rapidly and yeast adenylic acid yields  $\text{NH}_3$  at about 12 % of this rate. As the concentration is lowered and the pH shifts more closely to 8.3 the difference between the deamination rate for the two nucleotides becomes less until at a substrate concentration of 0.05 % nucleotide the  $\text{NH}_3$  is formed from both at the same rate. This can be satisfactorily interpreted only by supposing that with low concentrations and a pH of approximately 8.3 the pure adenylic deaminase is no longer involved, but that the two nucleotides are being dephosphorylated to adenosine, the latter then being deaminated to inosine. (The red corpuscle is rich in adenosine deaminase as shown in a subsequent section.)

Such a conclusion is further supported by the fact that in plasma at a pH of 8.3 (bicarbonate buffering) the two nucleotides were found to be deaminated at the same rate independently of their concentrations (under other conditions of buffering and reaction the plasma was found to behave very differently).

We may therefore represent the stages leading to the  $\text{NH}_3$  formation in shed blood (with escape of  $\text{CO}_2$ ) as follows:

Adenylpyrophosphoric acid  $\rightarrow$  adenyldiphosphoric acid + 1 phosphoric acid  
 $\rightarrow$  adenylic acid + 2 phosphoric acid  $\rightarrow$  adenosine + 3 phosphoric acid  $\rightarrow$  inosine  
 +  $\text{NH}_3$  + 3 phosphoric acid.

(b) *Blood reaction maintained at pH 7.4 by  $\text{CO}_2$ .* The laked blood was maintained at a pH of 7.4 by addition of bicarbonate (approximately  $N/40$  in mixture) and a tension of 35 mm.  $\text{CO}_2$ . Each tonometer used (about 300 ml. volume) contained 4 ml. mixture, the blood dilution therein being 1 in 5. A comparison of the rates of deamination of vegetable and muscle adenylic acids was made, and at all concentrations the vegetable adenylic was found to be deaminated at only a fraction of the rate (10–12%) of the muscle adenylic acid (as shown in Table V). Clearly the main path of the deamination is here through adenylic acid and not through adenosine.

*The "beta" phases.* When the course of formation of the "beta"  $\text{NH}_3$  is followed in rabbit blood at room temperature it occurs in a series of phases as shown in Fig. 9 (curve A). The same is observed in human blood when maintained at  $38^\circ$  (curve C) and also at room temperature in the fowl's blood (curve B).

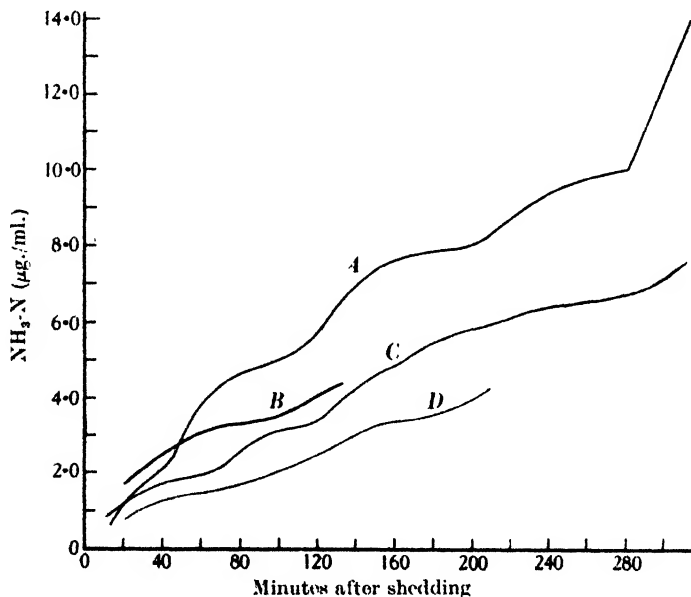


Fig. 9. Curves of ammonia formation in shed blood. A, rabbit blood at  $18^\circ$ ; B, fowl's blood at  $18^\circ$ ; C, human blood at  $38^\circ$ ; D, rabbit laked blood (dilution 1 in 2 at  $18^\circ$ ).

The curves in Fig. 9 are given as representative and not averaged from the data since this averaging tends to smooth out the inflexions owing to the appearance of these at somewhat different times from one animal to another. For the demonstration it is necessary to make about 30–40 consecutive determinations on the one blood specimen.

The  $\text{NH}_3$  formed in each phase is of the order of 2.0–3.0  $\mu\text{g.}/\text{ml.}$  The reality of these "beta" phases ( $\beta_1$ ,  $\beta_2$ , etc.) is very demonstrable from the fact that, at low room temperatures, the rise of the second phase may be interrupted for as long as 2 hr., the blood  $\text{NH}_3$  remaining constant (as in Fig. 11, curve C). In all

the rabbit blood examined the  $\beta_1$  phase was specially marked, and so far as we can judge is always easily demonstrable in a consecutive series. It begins in 10–20 min. after shedding and ends about the 50th min.

*Origin of the "beta" phases.* Possibilities such as sedimentation of corpuscles, phasic corpuscular permeability, the effect of sudden changes of reaction from

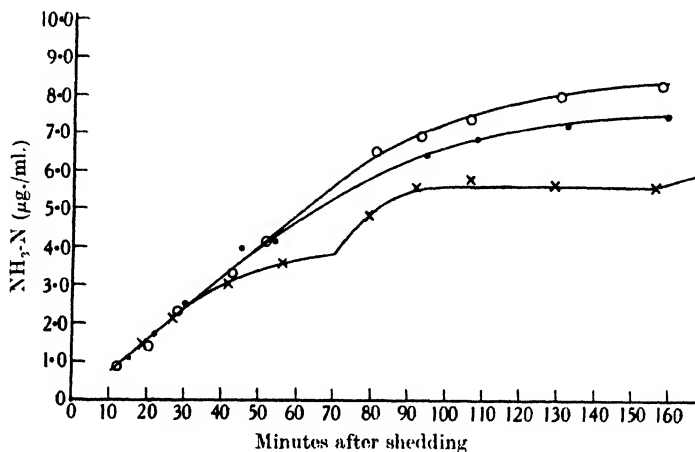


Fig. 10. The effects of fluoride and iodoacetate on the  $\text{NH}_3$  formation in rabbit's blood. Room temperature. Upper curve (circles), iodoacetate (1 vol.  $M/5$  to 20 vol. blood); middle curve (dots), fluoride (1 vol.  $M/5$  to 40 vol. blood); lower curve (crosses), control blood (1 vol. of  $M/5$  saline to 40 vol. blood).

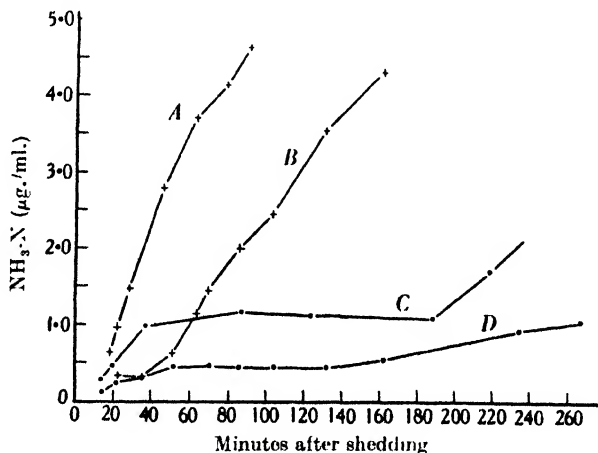


Fig. 11. Effect of adenylypyrophosphate addition to laked rabbit blood (total blood dilution, 1 in 2.45) pH of mixture, 8.0. A, adenylypyrophosphate addition (maximum  $\text{NH}_3\text{-N}$  69  $\mu\text{g./ml.}$ ); B, adenylypyrophosphate addition (maximum  $\text{NH}_3\text{-N}$  34.5  $\mu\text{g./ml.}$ ); C and D, blood controls for A and B. Blood laked and A.T.P. added immediately after shedding. Room temperature 12–13°.

unknown causes were eliminated; the blood being always shaken before an analytical sample was removed, and since the phases occur also in laked blood the question of permeability does not enter. That small changes in reaction are in themselves an immediate cause was excluded by treating the blood with alkaline phosphate ( $M/10$  concentration in mixture).

From a consideration of the stages of the breakdown of A.T.P. given above, it might be thought at first that these were related to the phasic deamination. What appears to be immediately involved however is the re-esterification of the formed adenylic acid and possibly adenyldiphosphate, because the action of iodoacetic acid or fluoride converts the "beta" formation into a smooth curve. This is shown in Fig. 10. Small amounts of the reagents were added to the blood so as to make a total  $M/200$  solution. The effect is a greater rapidity of  $\text{NH}_3$  formation after  $\frac{1}{2}$  hr. and a smooth curve. If such reagents are increased to  $M/50$ , or over, the opposed effect of a diminished rate is obtained. This we may explain by an inhibition of the initial dephosphorylation of the adenylypyrophosphate.

*Adenylypyrophosphate addition and the "beta"  $\text{NH}_3$*

Neutral sodium adenylypyrophosphate, prepared by Lohmann's method [1931] passing through the Ba salt, was added to laked blood producing an increase in the amino-N of  $34.5 \mu\text{g./ml. N}$ , the blood being 2.45 times diluted. In a second experiment adenylypyrophosphate was added in twice the previous amount. The course of the  $\text{NH}_3$  formation was investigated over 4 hr. and the results summarized in Fig. 11. It will be seen that in the first experiment (curves *B* and *D*) the adenylypyrophosphate produced no increase in the  $\text{NH}_3$  formation until about 50 min. had elapsed, when the curve rose abruptly. Twice the amount of added substance showed an earlier increase (curves *A* and *C*) and the curve of formation had a slope twice as steep.

With the laked blood without any addition (but diluted to the same extent) the  $\beta_2$  phase was very long delayed, but then appeared with a well-marked rise. The slow appearance of this phase could be attributed to the unusually low laboratory temperature of  $12-13^\circ$ .

In the blood therefore and also probably formed after the dephosphorylation in the breakdown of the A.T.P. there is a substance which either inhibits this breakdown or brings about a re-esterification.

As is well known from re-esterification in muscle extracts [Parnas *et al.* 1935], phosphopyruvic acid can bring this about and the prevention of the formation of phosphopyruvic acid by fluoride or iodoacetic acid will lead to a more rapid disappearance of the A.T.P. and increase in the  $\text{NH}_3$  formation. This is what we have seen to be the case on adding iodoacetic acid and fluoride ( $M/200$ ) to blood. It would be easier, however, to explain the phasic or cyclical  $\text{NH}_3$  formation if a substance actually inhibiting the breakdown of A.T.P. instead of re-esterifying were found. If we were to suppose such a substance effective above a certain concentration and that it was at the same time being continually removed, a brief consideration would show that it could bring about the "beta" phases under discussion.

Table VI

Blood mixture	Total A.T.P. present as $\mu\text{g./ml. amino-N}$ in mixture	$\mu\text{g./ml. NH}_3\text{-N}$ formed in	
		240 min.	1200 min.
I	$69 + 4 = 73$	16.2	32.0
II	$34.5 + 4 = 38.5$	6.4	15.8
Controls for I and II	None added $+ 4 = 4$	1.3	3.1

Fresh rabbit blood was laked and neutral adenylypyrophosphate added to I and II samples, similar volumes of 0.9 % saline being added to control samples. The blood dilution in the mixture was 1 in 2.45.

From the above experiments it appears also that A.T.P. increases the deamination rate in blood in proportion to its concentration in laked blood. Table VI

summarizes the data in this respect. The  $\text{NH}_3$  formation for the lowest concentration of A.T.P. in Table VI (blood to which no A.T.P. was added) is somewhat higher than might be expected from the linear relation, but under the conditions this will no doubt include in comparatively appreciable amount the "gamma"  $\text{NH}_3$  subsequently discussed.

*The pH effect on the "beta"  $\text{NH}_3$  formation*

This was examined for rabbit blood,  $M$  or  $M/2$  buffers being added to produce  $M/20$  concentrations in the total mixture, and the  $\text{NH}_3$  formation examined over several hours. The buffers used were maleic acid, phosphoric acid,  $\text{CO}_2$  on the acid side and bicarbonate and carbonate on the alkaline. The  $pH$  determinations were carried out either electrometrically on the plasma separated under paraffin or colorimetrically on saline equilibrated across collodion sacs containing blood.

The investigation was carried out from a  $pH$  of 7.0 to about 10.5. Fig. 12 summarizes numerous observations, the curves  $A$ ,  $B$  and  $C$  representing the 3, 2 and 1 hr. formations. The alkaline optimum lies between 8.5 and 9.0, though at 8.0, 9.1 or 9.2 it is very little less. Beyond 9.5 or 10.0 the  $\text{NH}_3$  becomes no doubt appreciably increased by the alkaline action on other sources of  $\text{NH}_3$ . This range of the optimum  $pH$  agrees with that for the breakdown of adenylypyrophosphate by liver extract, as shown by Barrenscheen & Lang [1932].

The  $pH$  effect on the blood  $\text{NH}_3$  formation was investigated by Parnas & Heller [1924] who found that it was reduced to a very low figure at a  $pH$  of 9.3 and they put the optimum at about 8.1. The difference between such results and those described are due to the use of borate buffers by Parnas & Heller. These have been shown to inhibit dephosphorylation [Hommerburg, 1935] and their effect may be thus indirectly explained in this fashion. The specific quality of the borate buffers in inhibiting the nucleotide has been subsequently recognized and used, e.g. by Heller & Klisiecki [1932].

The  $pH$  effect as found in such experiments and summarized in Fig. 12 demonstrates at least that the optimum deamination (on the alkaline side) occurs at the same or approximately the same  $pH$  as the alkaline phosphatase optimum—an expected result when we consider the original source as A.T.P. As shown in a subsequent section, the type of buffer is of the greatest importance in studying pure adenylic acid deaminations.

*Salt action.* Saline alone will inhibit the  $\text{NH}_3$  formation when added beyond a molar concentration of 0.1 and at 1.0  $M$  the influence is very marked.

*Defibrinated blood and the "beta"  $\text{NH}_3$ .* Defibrinated rabbit blood shows a very similar  $\text{NH}_3$  formation to oxalated blood. It is slightly faster at first and somewhat slower later, but is otherwise the same. The comparison was made on blood from the same rabbit (two experiments).

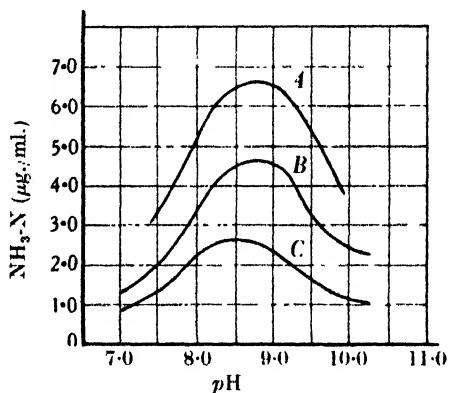


Fig. 12. The  $pH$  effect on rabbit's whole blood at room temperature 18°.

*The "gamma"  $\text{NH}_3$* 

This  $\text{NH}_3$  derives from a precursor in plasma and corpuscles and its formation is best studied in separated plasma in which no  $\text{NH}_3$  derives from A.T.P. or muscle adenylic acid after shedding.

The formation in plasma is very slow. After 24 hr.  $1.5 \mu\text{g./ml.}$  are present in human and a little over  $3 \mu\text{g./ml.}$  N in rabbit plasma. The figure for human plasma scarcely increases beyond that of the 24 hr. level, as shown when buffered with borate which acts also as a preservative. In rabbit plasma the  $\text{NH}_3$  formation is not ended in 24 hr., but goes on at reduced rates as the time advances. The following facts are significant in deciding what the precursor of this  $\text{NH}_3$  may be.

(1) *Specificity of deamination in plasma.* Deamination in plasma as in whole blood is extremely specific. As subsequently shown, out of 51 substances examined, of physiological significance and containing an amino or amine group, only adenosine or a few substances yielding adenosine by dephosphorylation are deaminated in mammalian blood after shedding, the  $\text{CO}_2$  being allowed to escape.

The substances capable of yielding adenosine by dephosphorylation include the two adenylic acids, and presumably A.T.P.

There are other possible combinations which can yield adenosine, for example, the co-ferment of Warburg, which however is present only in the red corpuscles and only to about 2 % of the total adenylic complex.

It is possible also that the "gamma"  $\text{NH}_3$  derives from the adenine deoxy-riboside which in turn may be formed from the corresponding nucleotide.

(2) *Elimination of free muscle adenylic acid or of A.T.P. as possible precursors of the "gamma"  $\text{NH}_3$ .* No free muscle adenylic acid or A.T.P. exists in mammalian plasma as shown by the action of voluntary muscle extract. This gives no increased formation of  $\text{NH}_3$ , three experiments of the following type being very carefully carried out. In these the blood from a rabbit was centrifuged at once, the plasma removed and the following tubes set up.

- (a) 2 ml. plasma + 0.5 ml. water + 0.1 ml. buffer.
- (b) 2 ml. plasma + 0.5 ml. extract + 0.1 ml. buffer.
- (c) 2 ml. water + 0.5 ml. extract + 0.1 ml. buffer.
- (d) 2.5 ml. water + 0.1 ml. buffer.

The buffer was 0.2 *M* neutral phosphate (6 vol. of 0.2 *M*  $\text{Na}_2\text{HPO}_4$  and 4 vol. 0.2 *M*  $\text{KH}_2\text{PO}_4$ ) and the muscle extract a 1 in 5 extract of voluntary muscle made up 3 hr. previously from another rabbit.

The tubes were left aside for about 1 hr. when the  $\text{NH}_3$  content was determined in each by triplicate analyses. The water used was  $\text{NH}_3$ -free and subtracting the trace of  $\text{NH}_3$  in (d) from that found in the other tubes, we get from (a) the  $\text{NH}_3$  in the plasma, from (b) the  $\text{NH}_3$  in plasma and extract after the action of the extract, and from (c) the  $\text{NH}_3$  in the extract. In exp. 1 of the group, the figures were  $0.62$ ,  $5.42$  and  $4.72 \mu\text{g. N/ml.}$  respectively, showing  $0.08 \mu\text{g. N/ml.}$  increase due to the extract, a quantity so small that it could arise as an analytical error. The two remaining experiments gave each a zero increase. In one of these the tubes were incubated for 2 hr. at  $38^\circ$ . Under similar conditions small amounts of added adenylic acid were deaminated in a small fraction of the time allotted for the above extract action.

(3) *Elimination of free adenosine as precursor.* Adenosine itself cannot be directly involved since it is deaminated too rapidly, small amounts disappearing in at most a few minutes in whole blood and being also deaminated with some rapidity in plasma.



(4) *Dephosphorylation and the pH effect.* From the foregoing evidence it would appear that likely precursors are vegetable adenylic acid, or alternatively the adenine deoxyribonucleotide. With either of these substances we may suppose a preliminary dephosphorylation with subsequent deamination of the nucleoside. At the pH reached by plasma after shedding with escape of  $\text{CO}_2$  vegetable and muscle adenylic acids yield  $\text{NH}_3$  at practically the same rate (Table VII) and this can be explained only by a preliminary dephosphorylation.

Table VII

Concentration of nucleotide in mixture	pH	NH <sub>3</sub> formation in plasma mixture over 24 hr., $\mu\text{g. N/ml. in}$		Control plasma mixture
		Muscle adenylic mixture	Yeast adenylic mixture	
Rabbit A. Bicarbonate buffering. Nucleotide mixture added at pH 8.3.				
0.1 bicarbonate in mixtures. Plasma dilution 1 in 2.5. Room temperature				
0.80	8.3	8.92	8.32	1.2
0.16	8.3	9.32	7.94	
0.04	8.3	9.32	9.82	
Rabbit B. Nucleotide buffering. Neutral adenyate added to plasma in equal volumes. Plasma dilution 1 in 2.0				
1.0	7.6 (at end)	35.0	5.8	1.8
Rabbit C. Nucleotide-maleic acid buffering. Maleic acid $M/20$ in mixture. Plasma dilution 1 in 2.5				
1.0	7.0	72.6	6.3	1.4
0.1	7.0	6.3	2.3	

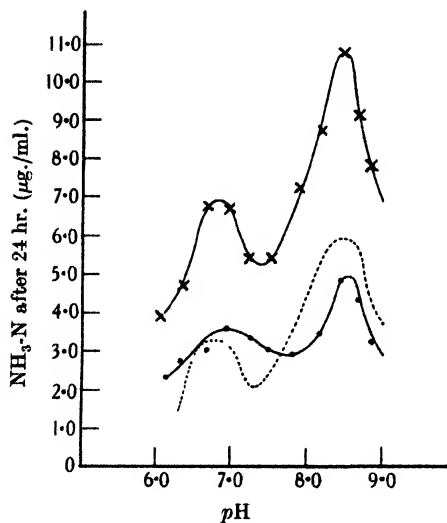


Fig. 13.

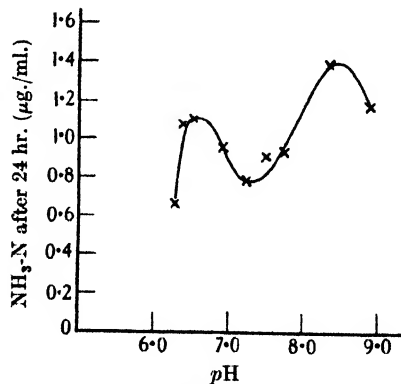


Fig. 14.

Fig. 13. The pH effect on the 24 hr. formation of  $\text{NH}_3$  in rabbit plasma (lower curve) and the effect of adding vegetable adenylic acid thereto (upper curve). The dotted curve gives the extra  $\text{NH}_3$  formed from the adenylic acid.

Fig. 14. The pH effect on the 24 hr. formation of  $\text{NH}_3$  in human plasma.

Assuming the substance to be vegetable adenylic acid and the deamination to follow on a preliminary dephosphorylation, then the curve of formation of the

"gamma"  $\text{NH}_3$  with respect to the  $\text{pH}$  of the buffer mixture may be expected to show two peaks, one about  $\text{pH}$  8.5-9.0 and the other somewhat to the acid side of neutrality. Such double peaking has in fact been very satisfactorily demonstrated with the peaks at 6.8 and 8.6 respectively (Figs. 13 and 14). Not only did the normal plasma  $\text{NH}_3$  appear with a double peaking when plotted against the  $\text{pH}$  but the extra  $\text{NH}_3$  formed after adding vegetable adenylic acid showed an almost identical curve with peaks in the same position (dotted curve in Fig. 13). The buffer mixture used consisted of a phosphate-citrate-borate solution containing equivalent amounts of the three substances and to which small varying amounts of  $N$   $\text{HCl}$  or of  $N$   $\text{NaOH}$  were added. To 1 vol. plasma 0.35 vol. of the buffer mixture was added to make approximately an  $M/10$  solution with respect to each buffer constituent.

After these experiments a careful examination was made of guanylic acid, cytidylic acid, guanosine and cytidine additions to plasma, for it seemed a possibility that these acids with their derived ribosides might be involved. Cytidine was prepared by the method described by Levene & Bass [1931]. The other substances (British Drug House preparations) were first purified from traces of  $\text{NH}_3$ .

Observations were carried out over 24 hr. and at a  $\text{pH}$  of 6.7—corresponding to the "acid" peak. Guanylic acid, guanosine and cytidine gave negative results. Cytidylic acid formed  $\text{NH}_3$  at a similar rate to vegetable adenylic acid. This was due to the fact that commercial cytidylic acid contains vegetable adenylic acid as an impurity. A purer product made in the laboratory gave rise to no  $\text{NH}_3$  as found in earlier experiments on whole blood.

(5) *The total organically bound acid-soluble phosphate in plasma and the increase of the free inorganic phosphate.* It is obvious that, if the "gamma"  $\text{NH}_3$  derives from vegetable adenylic acid or the adenine deoxyribonucleotide, organically bound "acid-soluble" phosphate must exist in corresponding quantity in plasma. It is estimated (e.g. Kay [1931]) that for dog's plasma this is about  $3\mu\text{g. P/ml.}$ , which corresponds approximately to  $1.4\mu\text{g. P/ml.}$  or an amount corresponding to the "gamma" ammonia in human plasma. We have carefully determined the acid-soluble organically bound phosphate in three rabbit plasmas, using the spectrophotometric method as described by Bomskov [1932]. Separate curves were used for the free inorganic and the total acid-soluble phosphate, constructed from standard phosphate brought through the whole procedure. The photometric readings were made by an independent observer. In these rabbit experiments (Table VIII) the mean inorganic phosphate was markedly higher

Table VIII

Rabbit	Free inorganic P in plasma $\mu\text{g. ml.}$		Total acid soluble P in plasma $\mu\text{g. ml.}$	
	Immediate	After 24 hr.	Immediate	After 24 hr.
I	69	72	75	76
II	74	77	84	84
III	73	75	78	78
IV	92	97	—	—
V, VI and VII	62	—	—	—

than that in normal human plasma, being  $72.0\mu\text{g. P/ml.}$  The total acid-soluble phosphate was  $79.3\mu\text{g. P/ml.}$  The organically bound P, being therefore  $7.3\mu\text{g. ml.}$ , would account for the first 24 hr. formation of  $\text{NH}_3\text{-N}$  in rabbit plasma (about  $3\mu\text{g. N/ml.}$ ).

It will appear also that if a nucleotide is being dephosphorylated before deamination that the free inorganic phosphate may be expected to show some increase over the 24 hr. period. In each of 4 exp. this increase was found, the mean value being  $3.2 \mu\text{g. P/ml.}$ —corresponding to  $1.5 \mu\text{g./ml.}$  Though this figure is under the expected amount if all the “gamma”  $\text{NH}_3$  proceeds from nucleotide dephosphorylation and subsequent deamination, yet it is quite possible that inorganic phosphate may decrease slightly by ester formation.

(6) *Rate of plasma  $\text{NH}_3$  formation and of adenylic acid deamination.* If vegetable adenylic acid is a source of the  $\text{NH}_3$  formation in plasma, then the rate of the extra deamination on adding yeast adenylic acid should correspond with the normal formation. As an example of this correspondence the following experiment may be cited. To 2 vol. of plasma 0.7 vol. of the citrate-phosphate-borate-buffer and 0.1 vol. of water were added producing a pH of 8.6 (alkaline optimum). A similar mixture was set up 0.1 vol. of 2 % sodium adenylate. In the first 4 hr. the  $\text{NH}_3$  was formed at a mean rate of  $0.35 \mu\text{g./ml. N/hr.}$  in the first mixture and at 0.40 in the second. In the next 20 hr. the mean rate in the first fell to  $0.14 \mu\text{g./ml. N/hr.}$ , but in the nucleotide mixture remained at 0.38. The obvious explanation here is that the concentration of the nucleotide in plasma already present was somewhat above the level at which the substrate concentration ceases to have effect, but as the deamination proceeds a point is reached at which the deamination rate is affected by the concentration and the  $\text{NH}_3$  formation falls. In the mixture with added nucleotide it continues unchanged.

(7) *Direct determination of adenine nucleotide in plasma.* If adenine nucleotide exists in plasma then the method of Buell & Perkins [1928] (recently modified by Buell [1935]) could be considered applicable to its determination, whichever modification it may be. In this method the blood nucleotide after deproteinization by trichloroacetic acid is precipitated as the uranium salt, the nucleotide being then hydrolysed to adenine by  $\text{H}_2\text{SO}_4$  and the adenine determined nephelometrically as the Ag salt—subsequent to the removal of the uranium by  $\text{NH}_3$ . The method was applied here to plasma. To 2 vol. of plasma 3 vol. of 13.3 % trichloroacetic acid were added, and the method carried out on each plasma in triplicate, including triplicate blank determinations and duplicate recoveries of added amounts of the vegetable nucleotide (of the same order as the expected content in plasma). The recoveries so far have been rather poor and variable (up to 60 %) but the existence of the nucleotide in plasma appears to be proved, in amounts which can be only provisionally stated as about 5 to 10 mg./100 ml.

Summarizing this evidence, one may say that the “gamma”  $\text{NH}_3$  in plasma is formed either altogether or in large part from an adenine nucleotide other than muscle adenylic acid, and that the formation occurs after a preliminary dephosphorylation. Vegetable adenylic acid fulfils the requirements so far considered for the precursor of the “gamma”  $\text{NH}_3$  in plasma.

#### IV. SUBSTANCES DEAMINATED AFTER ADDITION TO SHED BLOOD

A large number of substances containing an amino or substituted amino group was investigated for deamination on adding to shed blood. The object of this investigation was not to study the full possibilities of blood deamination, but rather to make clearer the sources of the blood  $\text{NH}_3$ . With this end in view the conditions established were never very far from those in shed blood, the pH range being from 6.7 to 8.3.

Apart from adenosine and high concentrations of muscle adenylic acid (both of which are rapidly deaminated by whole blood) the usual procedure adopted was to introduce into a sterilized vaccine bottle 10 vol. of laked blood (1:1) or of plasma with 8 ml. of thymol water and 3 ml. of neutralized substrate (with or without buffer). A small crystal of thymol was generally added as well. Very occasionally toluene was used instead of thymol. The bottle was capped and sealed with the usual vaccine technique, fluid for analysis being removed through a sterilized hypodermic needle introduced through the cap. The investigations were extended as a rule to 24 hr. or longer at room temperature (mean of 18°). The procedure was occasionally varied with regard to blood volume etc. The whole blood dilution throughout was from 4.2 to 2.5 times, and for plasma from 2.1 to 1.2 times. Two control bottles were always set up, the substrate being omitted from one and the blood from the other, the same volume and other conditions being maintained. The deamination of the substrate in the blood mixture was always reckoned by comparison with these controls.

In the expression of the resulting deaminations where positive results were obtained these are given as  $\mu\text{g. ml. N. min.}$  with respect to the original blood.

*Negative results* were obtained for mammalian blood with the following substances: arginine, alanine, aspartic acid, glycine, glutamic acid, *L*-histidine, ornithine, *L*-tyrosine, valine, proline, *L*-tryptophan, *L*-cystine, cysteine, glutathione, egg-white digest with pancreatic juice (boiled), acetamide, propionamide, asparagine, histamine, adrenaline, tyramine, acetylcholine, choline, colamine, betaine, neurine, glycoeyamine, glucosamine, guanidine, ethylguanidine, dimethylguanidine, creatine, creatinine, uric acid, vitamin B<sub>1</sub>, allantoin, guanine, adenine, cytosine, guanosine, cytidylic acid, guanylic acid, methylamine, ethylamine, propylamine, butylamine and isoamylamine.

*Positive results* for mammalian blood were obtained with adenosine, adenylic acid (muscle and vegetable), adenylypyrophosphate and thymus nucleic acid (slight action) as shown in Table IX. In fowl's blood guanosine, guanine, adenine, cytidine and cytosine were in addition deaminated rather freely.

In all cases of positive action sufficient time was left for the production of at least  $1\mu\text{g./ml. NH}_3\text{-N}$  and usually considerably more. This amounts to about 10 large divisions on the burette using the technique described. It will be seen that the deamination rate of adenosine is very much greater than that of adenylypyrophosphate, vegetable adenylic or muscle adenylic acid when this is at a level of 0.1 % (far exceeding any possible normal value). When the concentration of muscle adenylic acid is increased to as much as 1 % the deamination rate surpasses even that of adenosine. The significance of this is subsequently considered. The order of the adenosine deamination rate is also much greater than that of guanine, adenine etc. in fowl's blood. Guanosine is attacked much more rapidly than adenine, guanine, cytosine and cytidine in fowl's blood, and guanosine deaminase also exists in the fowl's plasma.

Concerning the sources of the substances used in the above investigations, adenylypyrophosphoric acid was prepared by Lohmann's method [1931], muscle adenylic acid by that of Ostern & Parnas [1932], later supplies being received from Messrs Henning, Berlin. Cytidylic acid, cytidine and cytosine were prepared by the methods of Levene & Jacobs [1911], Levene & Bass [1931] and Levene [1903]. Ornithine, betaine and neurine were supplied by Dr Schuchardt, Görlitz, and the remaining substances by the British Drug Houses.

Table IX

Substance	Subject	Blood used	Substrate conc. %	$\mu\text{g. N/ml./min.}$ ( $\times$ dilution)	pH	Buffer	Temp.
Adenosine	Man	Whole	0.2	1.25 (4)	7.0	Phos.	18
		Plasma	0.2	0.23 (1)	6.7	Maleic	38
	Rabbit	Whole	0.2	5.85 (5)	7.0	Phos.	18
		Plasma	0.3	0.040 (1)	7.0	Maleic	18
	Fowl	Whole	0.03	6.590 (2)	7.0	Phos.	21
		Plasma	0.03	0.007 (1)	7.3	Phos.	25
	Frog	Corpuscles	0.1	6.040 (6)	6.7	Maleic	16
		Plasma	0.1	0.550	6.7	Maleic	16
	Arenicola	Whole	0.03	0.058 (3)	6.7	Maleic	16
Adenylic acid (muscle)	Man	Whole	1.0	3.480 (1)	7.0	Maleic	18
			0.05	0.066 (1)	7.0	Maleic	18
	Rabbit	Whole	1.0	8.900 (2)	7.0	Maleic	18
			0.1	0.020		Maleic	18
		Plasma	1.0	0.126 (1)	7.0	Maleic	18
			0.1	0.011			
			1.0	0.014 (2)	8.3	Bicarb.	20
			0.1	0.014	8.3	Bicarb.	20
Adenylic acid (yeast)	Rabbit	Whole	1.0	0.80 (2)	7.0	Maleic	21
			0.1		7.0	Maleic	21
			1.0	0.748 (2)	8.3	Bicarb.	19
			0.1	0.037	8.3	Bicarb.	19
		Plasma	1.0	0.011 (1)	7.0	Maleic	18
			0.1	0.004 (1)	7.0	Maleic	18
			1.0	0.013 (2)	8.3	Bicarb.	20
			0.1	0.015	8.3	Bicarb.	20
Adenylpyro- phosphoric acid	Rabbit	Whole	0.28	0.169	8.0	No extra	19
			0.15	0.065 (2)	8.0	Buffer	19
			0.015	0.013	8.0		19
Nucleic acid (thymus)	Rabbit	Whole	0.3	0.004 (2)	8.0	No extra	18
			0.3	0.010 (1)	8.0	Phos.	40
			0.3	0.002 (1)	8.0	Phos.	18
Adenine	Fowl	Whole	0.04	0.029 (1)	7.3	Phos.	21
			0.04	0.038 (1)	8.0	No extra	21
Guanine	Fowl	Whole	Sat.	0.060 (1)	7.3	Phos.	21
Cytosine	Fowl	Whole	0.2	0.088 (1)	7.3	Phos.	21
Guanosine	Fowl	Whole	Sat.	0.309 (1)	7.3	Phos.	25
		Plasma	Sat.	0.009 (1)	7.3	Phos.	25
Cytidine	Fowl	Whole	0.1	0.034 (1)	8.0	No extra	21
Adenine- cytosine dinucleotide	Fowl	Whole	0.1	0.014 (1)	7.3	Phos.	25

The bracketed data were obtained from the same blood. The bracketed numbers give the total animals. Buffer strengths ranged from 0.1 to 0.2 *M*.

### DISCUSSION

Since small amounts of  $\text{NH}_3$  are probably liberated continually into the blood stream it may be more correct to hold that the characteristic value of the blood  $\text{NH}_3$  is below the analytical level (about 0.002–0.003 mg./100 ml.  $\text{NH}_3\text{-N}$ ) than that it is zero; though as previously noted [Conway, 1935] the extrapolation of the mean curve formation gives no free  $\text{NH}_3$  at zero time.

Among the more reliable results obtained by other observers the nearest to this was found by Markert [1934; 1936] using a slight modification of the

Parnas-Heller method in which he determined the  $\text{NH}_3$  spectrophotometrically. Markert placed the  $\text{NH}_3$  content in human blood at less than 0.010 mg./100 ml.  $\text{NH}_3\text{-N}$  and in 30 experiments found no trace of free  $\text{NH}_3$  therein

For the first or "alpha" rise after shedding—occurring in the first 5 min. and amounting to about 2 % of the total  $\text{NH}_3$  that forms—we have given evidence to show it may derive from adenosine. For this, however, it is necessary to suppose that adenosine in minute concentrations is in some way protected by  $\text{CO}_2$  from the action of the ferment. This so far has not been demonstrated, though not fully investigated. On the other hand, an undoubted and powerful inhibition by the  $\text{CO}_2$ -bicarbonate system has been shown for adenylic acid deaminase, so that the possibility must be considered of minute amounts of free adenylic acid causing this "alpha" rise. The difficulty of explanation here would be one of rate of action. Reckoning from the effect on adenylic acid added to laked blood, about 20 % at most of minute amounts of adenylic acid are deaminated in 5 min. in unchanged blood, and this is derived from a supposition of linearity of action from high substrates downwards. As we have seen the indication is that at the lowest levels the slope of the curve is less. A further factor, however, must also be considered, namely, that the blood immediately after shedding has a temperature not far from  $37^\circ$  and within the first few minutes the deaminating action is no doubt very considerably greater than at  $18^\circ$ . It remains possible, therefore, that the "alpha"  $\text{NH}_3$  derives from a minute amount of free adenylic acid.

However this may be, for the "alpha"  $\text{NH}_3$  it is certain that the "beta"  $\text{NH}_3$  derives from the breakdown of adenylypyrophosphate. In shed blood with an escape of  $\text{CO}_2$  dioxide the path has been shown to pass through adenosine, but the deamination is mainly direct under normal conditions. This  $\text{NH}_3$  amounts to about 1.0 mg.  $\text{NH}_3\text{-N}$  100 ml. both for the rabbit and the human subject, and accounts for 60–75 % of the whole formation. Attention may be drawn here again to the curious phasic appearance of the "beta"  $\text{NH}_3$  and its apparent relation to re-esterification processes. The first of these phases is the most characterized and it is only after this phase has ended that added A.T.P. produces extra  $\text{NH}_3$  unless added in large quantities.

*The "gamma"  $\text{NH}_3$ .* This is at present considered for plasma alone, though it probably also forms in the red corpuscle. Evidence has been presented that this derives from vegetable adenylic acid or possibly from the deoxyribonucleotide. Assuming the precursor to be vegetable adenylic acid it is the only one of the four adenylibose compounds, including adenosine, muscle adenylic acid and A.T.P. which can remain for any appreciable time in plasma. Adenosine disappears in the fraction of a minute (it is freely permeable through the red corpuscle) and muscle adenylic acid very probably in the fraction of a second as it passes through a voluntary muscle zone, for voluntary muscle is freely permeable to muscle adenylic acid, and presumably to adenylypyrophosphoric acid.

Consequently as a carrier of adenylibose grouping to tissues and as a comparatively durable substance with the pharmacological action of its class its significance may be considerable.

#### SUMMARY

1. A revised method is described for determining the blood  $\text{NH}_3$ .
2. The immediate or "alpha" rise of  $\text{NH}_3$  after shedding (about 0.04 mg. N/100 ml.) is dependent on the escape of carbon dioxide and not on a pH change. Evidence is given for its deriving from minute amounts of adenosine or adenylic acid.

3. Following the "alpha" rise in rabbit blood there is a comparatively rapid formation of  $\text{NH}_3$  in a series of stages ending in 3–5 hr. at room temperature and deriving from A.T.P. The path is largely through adenosine in shed blood with escape of  $\text{CO}_2$ , but mainly from adenylic acid if the  $\text{CO}_2$  tension be maintained. This  $\text{NH}_3$  in rabbit blood amounts to 50–60 % of the total forming in sterile blood (about 2.0 mg. N/100 ml.). In human blood it amounts to 80 % of this (1.3 mg. N/100 ml.) and forms much more slowly, 50 % only appearing after 24 hr. at room temperature. The optimum pH of its formation is 8.7.

4. An easy method has been described for determining the A.T.P. in shed blood very suitable for serial determinations, and agreeing with the much longer method of Parnas & Lutwak Mann [1935] through the Ba salt.

5. The "beta" phases of which the most marked is the first are abolished by iodoacetic acid or by fluoride (N/200).

6. A.T.P. added to shed rabbit's blood develops  $\text{NH}_3$  comparatively rapidly and in proportion to the amount added.  $\text{NH}_3$ , however, does not begin to form therefrom (unless large amounts have been added) until after the first "beta" phase.

7. The plasma or "gamma"  $\text{NH}_3$  (probably also forming in the red corpuscles) and amounting to about 1.5 and 4 mg. N/100 ml. in rabbit and in human blood respectively, appears to derive largely from yeast or vegetable adenylic acid (or possibly from the deoxyribonucleotide).

8. Of 51 substances of physiological importance containing amino or volatile groups, only the following yielded  $\text{NH}_3$  in blood: adenosine, adenylic acid (muscle), adenylic acid (yeast), A.T.P. and thymus nucleic acid—this last giving only minute amounts.

In avian blood adenine, guanine, guanosine, cytosine and cytidine are freely deaminated.

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# LIX. THE DEAMINASES OF ADENOSINE AND ADENYLIC ACID IN BLOOD AND TISSUES

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IN this paper an account is given of the activity of these enzymes as they appear in blood and tissue extracts. Whereas the deaminase of adenosine acts therein as one may expect, that of adenylic acid has certain unusual characteristics. The most striking fact is the great susceptibility of the adenylic deaminase to the inhibiting action of certain anions, though the anions of maleic and citric acids are practically without effect: besides which, there are other inhibiting substances in tissues (probably anions also) but not in voluntary muscle. The result of their action is a protection of the adenylic acid as it forms in tissues generally and a simulation of a proportionality of action to substrate concentration. From such effects also it may be occasionally concluded that adenylic acid deaminase is absent or in very small concentration when in fact very appreciable amounts are present.

From this study, voluntary muscle appears as a very exceptional tissue in so far as the mean *effective* deamination rate of adenylic acid therein would appear to be of the order of 500–1000 times greater than that in other tissues. The adenosine deaminase behaves very differently and its action is practically independent of the buffer used provided that the *pH* be maintained.

The distribution of the enzymes has been studied for 36 tissues of the rabbit with results of interest: it being shown, for example, that the appendix contained the highest concentration of adenosine deaminase in the group studied and presumably in the whole body.

As regards the course which the deamination of adenylic acid takes in tissues, it is shown that not only in voluntary muscle, but also in brain, nerve tissue, the auricle of the heart and blood maintained at normal *pH* the deamination is direct, but that in six other tissues examined and probably in the remaining tissues of the body, the deamination occurs only after a preliminary dephosphorylation.

The statement in the preliminary report in *Nature* [1938] that plasma was found to contain no pure adenylic acid deaminase is now revised, since further experiments have shown that it may contain small variable amounts, but these are of the order of 0.005 % of that present in skeletal muscle. This has appeared from the study of high nucleotide concentrations (buffered at *pH* 7.0) the enzyme being practically inoperative at low concentrations.

## PROPERTIES OF THE DEAMINASES IN BLOOD AND TISSUES

**Methods.** Water extracts of tissues ground with pure quartz sand (Merck's) were used throughout unless otherwise stated. The efficiency of extraction with water and saline extracts etc. is commented on below. For the ammonia and other estimations the procedure described in the previous paper [Conway & Cooke, 1939] was used.



*Time and enzyme activity*

Here we may distinguish at least two effects, firstly that on the enzyme-substrate activity and secondly the ageing or deterioration of the enzyme.

For adenosine deaminase acting for a few hours at room temperature and provided that the substrate concentration does not fall below  $5\mu\text{g./ml. amino-N}$ , the action shows a linear relation with time. The same applies for the adenylic acid deaminase when the action is negligibly small compared with the substrate concentration.

The activity of the extracts is found to decrease but little over 24 hr. at room temperature, but the deaminase of adenylic acid rapidly deteriorates at  $38-40^\circ$  (unbuffered or buffered at  $\text{pH } 7.0$ ) giving only a small fraction of its activity after a few hours. Similar observations at the higher temperatures have not been made with adenosine.

*Substrate concentration*

*Adenosine.* The enzyme in laked blood and tissue extracts acts independently of the substrate concentration beyond a certain very small concentration level. From the data in Table I this level is from 2 to  $5\mu\text{g./ml. amino-N}$  for a 1 in 5 dilution of rabbit blood. The activity is proportional to substrate concentration when this is below *ca.*  $2\mu\text{g./ml. amino-N}$  or about 0.003 % of adenosine.

Table I

Adenosine concentration in mixture at zero time	Deamination in 1 min.	% of full deamination after long period
Amino-N $\mu\text{g./ml.}$	$\text{NH}_3\text{-N}$ $\mu\text{g./ml.}$	
0.62	0.35	56.4
1.15	0.55	47.6
2.88	0.98	34.1
5.75	1.20	20.9
14.8	1.22	8.4
29.6	1.20	4.1

Blood dilution 1 in 5. Maleic acid buffer ( $M/20$ ) at  $\text{pH } 6.8$ .

*Adenylic acid (muscle).* The effect of the substrate concentration is here very different. With laked blood and dilutions of 1 in 5 to 1 in 50 and a  $\text{pH}$  of *ca.* 7.0 the typical course of the deamination with increase of substrate is shown in Figs. 1 and 2. In such experiments with either human or rabbit blood the curve shows three sections. In the first comparatively short stage the increase of deamination rate with substrate is much less than in the second, beginning at about 0.1–0.2 %. From this on to 1 % and more the course is strictly linear. At or beyond 1 % it bends to reach saturation rate of the deamination. The same features of the curve may be observed independently of the buffer used, but the actual rate of deamination will be found to vary as one buffer or another is employed. Even without any added buffer (except the adenylyate) the upper parts of the curve can be followed at comparatively steady  $\text{pH}$  (using short observation times) and found to correspond with the above description.

As shown below, about 90 % of the deamination is here proceeding by direct deamination of the adenylic acid and not after a preliminary dephosphorylation.

At the saturation level of the adenosine deaminase (about 0.005 %) the  $\text{NH}_3$  formation from adenosine is *ca.* 1000 times greater than from adenylic acid, but at *ca.* 100–200 times this concentration the rate approaches that of adenosine and

finally considerably exceeds it. The physiological action will be confined to the lowest concentration levels.

The effect of the substrate concentration in extracts of six different tissues is shown in Fig. 3. The dilution was 1 in 40 with maleic buffering at pH 7.0. The

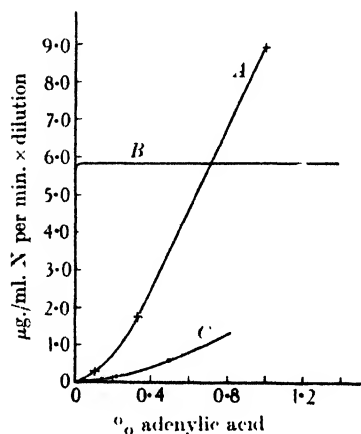


Fig. 1.

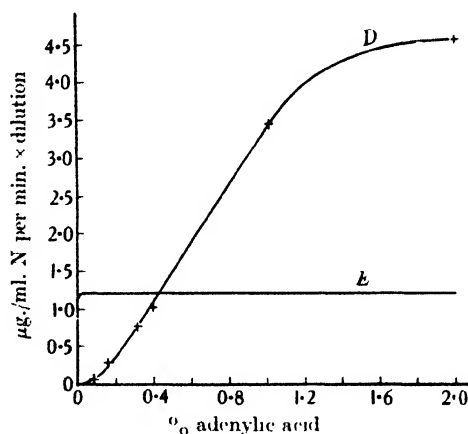


Fig. 2.

Figs. 1 and 2. Deamination rates of m. adenylic acid and of adenosine with respect to substrate in rabbit and human blood. *A*, curve of m. adenylic acid deamination in rabbit blood, with maleic acid buffer at pH 7.0. *B*, similar to *A* with phosphate buffer. *C*, curve of adenosine deamination with phosphate buffer, at pH 7.0. *D*, curve of m. adenylic acid deamination in human blood as in curve *A*. *E*, curve of adenosine deamination for human blood as in *C*.

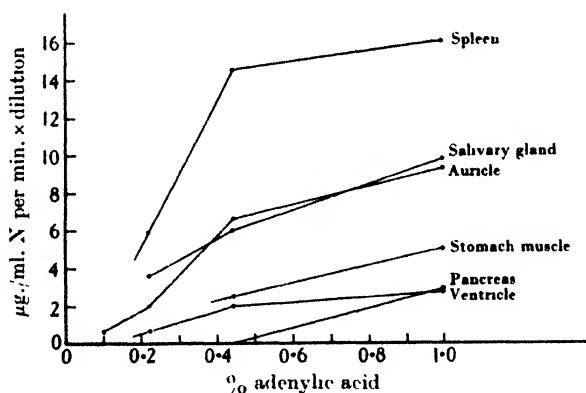


Fig. 3. Deamination of m. adenylic acid in tissue extracts. Maleic acid buffering at pH 7.0. Tissue dilution 1 in 50.

general effect is similar to that with blood. Voluntary muscle extract is markedly exceptional to this, the deamination rate increasing by only 10 % over the range 0.08–0.8 % and the mean value being over 70 times greater than the highest recorded in Fig. 3. Of the six tissues, however, only three deaminate the nucleotide in the main directly—these are voluntary muscle, cerebral cortex and the auricle of the heart. The others—as shown below—deaminate it almost if not quite entirely after an initial dephosphorylation, but apart from the extract of voluntary muscle the effect of substrate concentration is similar. From Fig. 3 it will appear

that at possible physiological levels—at and less than 0.1 % of the nucleotide the effective deamination rate in voluntary muscle extract is of the order of 500–1000 or more times as great as in other tissues.

Over the linear region of action of the enzyme with respect to substrate it may be pointed out that a unimolecular reaction relation will be simulated, this relation implying a proportionality of rate to substrate concentration. The underlying cause of the linearity is however quite different. (This does not contradict our previous statement of linearity of action with the time applying when the  $\text{NH}_3$  formed is negligibly small compared with the possible total.)

#### *The effect of dilution*

With the adenosine deaminase the effect is the expected one. The total deamination rate for 1 ml. blood or 1 g. tissue is independent of the volume in which this is dissolved or dispersed. It is quite otherwise with the adenylic acid deaminase in blood and other tissues. Here the deamination per 1 ml. blood increases greatly with the dilution if the substrate concentration is not very high. This is illustrated in Fig. 4 in which dilution and deamination are expressed logarithmically.

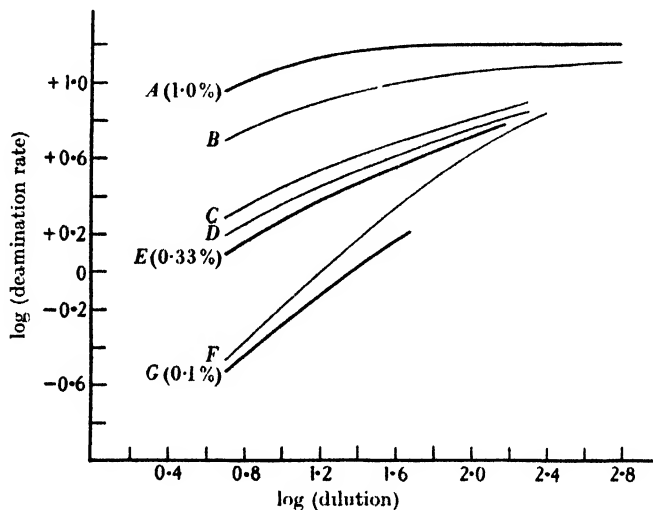


Fig. 4. Effect of dilution on m. adenylic acid deamination in blood. *A*, *E* and *G* are curves for same rabbit blood using 1, 0.33 and 0.1 % nucleotide and  $\text{CO}_2$  and bicarbonate buffering at pH 7.0. *B*, 0.5 % nucleotide, self-buffering. *C*, 0.5 % nucleotide,  $\text{CO}_2$  and bicarbonate buffering. *D*, 0.36 % nucleotide, self-buffering. *F*, 0.40 % nucleotide, malic buffering. All at pH 7.0.

The ordinates in the graph give the logarithms of the deamination rates ( $\mu\text{g./ml./min.}$ ) for 1 ml. rabbit blood independently of the volume in which it is contained. The thick lines *A*, *E* and *G* for nucleotide concentrations of 1.0, 0.33 and 0.1 % respectively are for the same rabbit blood with  $\text{CO}_2$  dioxide and bicarbonate buffering (*ca.* pH 7.0). The other curves are for other blood samples and different conditions of buffering as described under the figure.

It will be seen that the general effect in which dilution causes an ultimate approach to approximately  $13.8 \mu\text{g./ml. NH}_3\text{-N/min.}$ , is independent of the substrate concentration. With high substrates this is quickly reached, and with low after much dilution. *Dilution at the lower substrate levels can increase the total deamination 60 times or more.*

Maleic acid buffering in blood at comparatively small dilutions appears to have an inhibiting effect on the deamination and this would appear to be associated with some precipitate formation. For tissue buffering in general with the adenylic acid deaminase it is, however, much superior to phosphate, bicarbonate/ $\text{CO}_2$  etc. as will appear in the next section.

### *Specific buffer action*

While the ideal procedure would here require the purest enzyme preparations, yet direct examination of very dilute voluntary muscle extracts gives the essential information. When this dilution amounts to 2000 times, the extract is still very active, and provided that the correct conditions of room temperature, buffering and  $p\text{H}$  are present a rate of  $0.5 \mu\text{g. N/min.}$  is obtained which will produce  $\text{NH}_3$  in somewhat over  $\text{N/500}$  strength within 1 hr. In this way we examined five buffers, citric, maleic and phosphoric acids,  $\text{CO}_2$ /bicarbonate and veronal. The muscle extract dilution in the mixture was 1 in 2000, the buffer strength about  $M/20$  (the bicarbonate,  $\text{CO}_2$ ,  $M/40$ ). The  $p\text{H}$  was carefully fixed at 7.4 and the deaminating action not allowed to proceed for longer time than required for formation of  $\text{N/5000 NH}_3$ . The effect on adenosine deaminase of the same buffers was studied in a similar way using laked blood (total dilution of one in eight). Table II shows the results for 0.1 and 1.0 % of the nucleotide and corresponding

Table II

Buffer mixture at $p\text{H } 7.4$	Exp. no.	Adenylic acid deamination in 1 in 2000 voluntary muscle extract $\mu\text{g. N ml. min.}$ ( $\times$ dilution)			Exp. no.	Adenosine deamination in 1 in 8 diluted blood $\mu\text{g. N ml. min.}$ ( $\times$ dilution)		
		1 % nucleo- tide	0.1 % nucleo- tide	Ratio %		0.7 % adenosine	0.07 % adenosine	Ratio %
Maleic	1	150	97	65	5	2.54	2.49	98
	2	696	602	86	—	—	—	—
Citric	1	352	289	82	—	—	—	—
Phosphate	1	143	3	2	6	2.71	2.56	94
$\text{CO}_2$ /bicarbonate	3	350	3	1	6	2.64	2.82	107
	4	716	37	5	—	—	—	—
	1	42 (?)	3	—	—	—	—	—
Veronal	1	327	14	4	6	2.59	2.70	104

The strength of the buffers in the mixtures was from  $M/20$  to  $M/10$ .

concentrations of the nucleoside. It will be seen that a change of adenylic acid concentration from 1.0 to 0.1 % affects only to a small degree the deamination with maleic and citric buffers. With maleic buffer in two experiments the reduction was to 86 and 65 % (with this we may include the effect with an extract dilution of 40, when the reduction was to 90 %). With citric buffer the reduction was to 82 %. With phosphate, bicarbonate and veronal buffers on the other hand, the deamination rate falls almost to zero or less than 3 % in the mean. Apart from its theoretical significance it is of some practical analytical importance to realize that by a suitable choice of buffer the deamination rate of adenylic acid by dilute muscle extract may be increased to over 20–30 times.

If we consider the deamination rate at 1 % nucleotide of the various buffers, citric seems the most efficient with a rate of  $352 \mu\text{g. N/ml./min.}$  In exp. 1 the bicarbonate system gave the very low figure of 42, which may have arisen from

the use of 5 %  $\text{CO}_2$ /oxygen mixture from a cylinder, alveolar air being used in the other two experiments, though we cannot say why this difference should arise.

With adenosine deamination the results are very different. Here the rate is independent both of the substrate concentration (over the range studied) and of the buffer used.

The dilution was chosen so that the actual deamination rates in the blood-adenosine and the 1 %-adenylic-muscle-extract mixtures were not very different having means of 0.2 and 0.3  $\mu\text{g. N/ml./min.}$  respectively.

#### *Specific inhibitors of adenylic acid deaminase in blood and tissues*

The mechanism whereby a linear proportionality of deamination to substrate concentration is simulated is obvious enough from the above study. Very probably the anion of the special buffer is absorbed on to the enzyme system displacing the adenylic acid. Over a certain range a proportionality effect can be simulated on increasing the nucleotide concentration, particularly if this is not adsorbed as readily as the buffer.

The practical independence of substrate concentration seen in the muscle extracts with maleic or citric buffers and in a 1 in 40 as in a 1 in 2000 tissue dilution, does not appear in blood or general tissue extracts in dilutions up to 1 in 50. Here the bicarbonate and free phosphate concentrations are negligible (in a special blood experiment the bicarbonate was totally removed) yet the type of curve shown in Figs. 1 and 2 remains. Some other inhibitors are present which, as may be expected, are progressively weakened in effect by dilution as shown in Fig. 4. These inhibitors appear to be generally distributed in tissues as may be judged from Fig. 3 and may act in a similar way here on dephosphorylation as on deamination processes since vegetable adenylic acid in blood shows a somewhat similar curve on a much lower level; also in most of the tissues in Fig. 3 a dephosphorylation actually precedes the deamination (at least to a very large extent).

By shifting the  $p\text{H}$  of diluted blood towards 6.0–6.5 with citric buffer, the effect of the inhibitor is much weakened, the activity at 0.1 % nucleotide being 62 % of that at 1.0 %. The indication is that the inhibiting substance is an anion of a weak acid with  $pK$  value in the region of 7.0 and may be possibly a protein anion or anions. In voluntary muscle extracts such inhibitors have no appreciable effect in reducing the activity of the enzyme.

The question arises, however, how far do the normal  $\text{CO}_2$  and bicarbonate concentrations in muscle affect the adenylic deaminase. As shown, in very weak dilutions (1/2000),  $M/40$  bicarbonate and 38 mm.  $\text{CO}_2$ , the effect is very great. With stronger extract (1 in 40), 80 mm.  $\text{CO}_2$  and  $M/40$  bicarbonate the effect is much less, the deamination of 0.1 % nucleotide being 40 % of that of the 1 % solution. It may be presumed that with the very high enzyme concentrations in the tissue itself and a reaction around  $p\text{H}$  7.0, the effect is not marked.

#### *The pH effect*

*Adenylic acid.* The effect is shown in Fig. 5 where the activity is given in terms of a 100 value at  $p\text{H}$  7.0. The  $p\text{H}$  buffers used were maleic acid below  $p\text{H}$  7.0 and bicarbonate above, the strengths being *ca.*  $M/20$ ; a nucleotide concentration of 0.25 % was used in the mixture and blood dilutions of 1 in 40 (higher nucleotide concentrate would have been somewhat more satisfactory on the alkaline side). The slope of the curve on each side of  $p\text{H}$  7.0 was determined and the values at 7.0 regarded as 100.

The curve does not show the sharp-peaked optimum close to 6.0 described previously by Schmidt [1928]. It may be noted at the same time that the question of specific buffer inhibition was not studied by this worker.

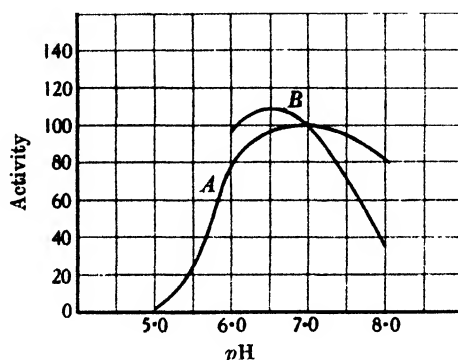


Fig. 5. Effect of pH on the deamination rate of m. adenylic acid (curve A) and of adenosine (B) in laked rabbit blood.

*Adenosine.* The adenosine curve was determined with phosphate buffers and 0.2–0.3 % adenosine. It does not differ appreciably from the results obtained using purified enzyme preparations [Schmidt, 1928].

#### THE PATH OF THE DEAMINATION OF MUSCLE ADENYLIC ACID IN TISSUES

The question here is what proportion of the deamination proceeds directly from the nucleotide or after an initial dephosphorylation. It may be considered for adenylic acid added to extracts, or for deaminations proceeding normally from the nucleotide complex. The study of the inorganic phosphate is not of much value for this purpose since phosphate may be transferred to other substances directly or after an initial liberation. We have used two deamination methods, however, whereby the question has been satisfactorily dealt with.

*First method.* In this method the deamination rate is examined after adding adenylic acid, after adding adenosine alone (enough to saturate the enzyme, which will occur after very small additions) and after adding adenylic acid plus adenosine. The difference between the deamination rates in the second and third mixtures will give the true or direct nucleotide deamination. Such a method will also give a measure of the true adenylic deaminase.

The method is not of immediate value in determining the path of normal deaminations owing to the much greater rate in general of adenosine deamination as compared with that of small added amounts of adenylic acid.

*Second method.* In this method the deamination rates of similar concentrations of yeast and muscle adenylic acids are studied in the same tissue extract or laked blood mixture.

Assuming that vegetable adenylic acid is not directly deaminated in animal tissues and that the enzymic dephosphorylation rates of both acids are the same (with acidic hydrolysis the yeast adenylic acid goes faster), we obtain the amount of direct deamination of the muscle nucleotide by the difference in deamination rates.

As regards the first point it has been shown by Schmidt [1928] that the purified enzyme from muscle is extremely specific and does not deaminate the vegetable nucleotide at an appreciable rate, and this we have also found for voluntary

muscle extract at room temperature (vide Table VI). In blood we have found that after elimination of dephosphorylation activity by dialysis the deamination rate of vegetable adenylic acid (in 1 % strength) falls almost to zero when examined over 24 hr., whereas that of the muscle adenylic acid remains practically unchanged. This is seen by comparing the two last columns of Table III.

Table III

	Mixture	Phosphate $\mu\text{g./ml. P}$ after		$\text{NH}_3$ $\mu\text{g./ml. N}$ after	
		2 hr.	20 hr.	2 hr.	20 hr.
Undialysed	Blood control	34.3	46.0	1.8	5.3
	M. adenylic mixture (0.8 %)	29.9	30.7	124.0	215.0
	V. adenylic mixture (0.8 %)	41.8	64.0	9.3	32.3
Dialysed	Blood control	1.4	1.6	0.7	0.3
	M. adenylic mixture (0.8 %)	1.8	1.8	120.0	192.0
	V. adenylic mixture (0.8 %)	1.4	0.0	4.5	4.7

The blood dilution in mixture was 1 in 4.2, pH 8.0-8.3.

Table IV

Tissue	M. adenylic acid deaminated $\mu\text{g. N/ml./min.}$	V. adenylic acid deaminated $\mu\text{g. N/ml./min.}$	True nucleotide deaminated II minus III $\mu\text{g. N/ml./min.}$
Blood	12.2	0.6	11.6
Auricle	13.3	2.7	10.6
Salivary gland	14.3	8.8	5.5
Jejunum	4.7	2.3	2.4
Stomach muscle	6.1	1.5	4.6

1 in 20 water extract of tissues were used. 1 vol. of extract plus 1 vol. of 2 % adenylic acid at pH 7.0. No other buffering used. Room temperature. The deamination rates are reckoned for the original tissue.

Table V

Tissue	Adenosine deaminated		Adenosine plus m. adenylic acid deaminated 0.16 % adenosine + 0.8 % m. adenylic acid in mixture	True nucleotide deaminated $\mu\text{g. N/ml./min.}$
	0.16 % $\mu\text{g. N/ml./min.}$	0.32 % $\mu\text{g. N/ml./min.}$	$\mu\text{g. N/ml./min.}$	
Blood	4.15	4.15	17.0	12.9
Auricle	6.3	6.7	17.1	10.6
Salivary gland	15.5	15.8	21.6	6.0
Jejunum	54.8	57.3	60.4	4.4
Stomach muscle	1.74	1.69	5.1	3.4

Similar conditions to those for Table IV, but maleic acid (*M*/20) used for buffering at pH 7.0.

As regards the second point—the equality of dephosphorylation of the two nucleotides—perhaps the best proof of this in the tissues generally is a comparison of the results from the two methods. This comparison is given in Tables IV and V for five tissues. The correspondence is quite good, especially when it is considered that analytical inaccuracies will have a big effect in the results from the salivary gland and jejunum extracts. Table III shows how little can be deduced from direct studies of the free phosphate.

With this second method we can examine the effect of adding amounts of each nucleotide to tissue extracts of a similar concentration to the nucleotide in the original tissue and so gain information as to the normal path.

For 11 tissues studied in this way (Table VI) voluntary muscle, the cerebral cortex, conducting nerve, auricle of heart and blood (under normal conditions) show direct deamination as the major path (75–90 %).

In the second group—which is sharply demarcated from the first—the deamination proceeds almost if not quite entirely by the indirect route. This group includes the ventricular muscle of the heart, stomach muscle, salivary gland, kidney, liver and jejunum. At high nucleotide concentrations apparently an appreciable fraction goes directly but as the nucleotide concentration is dropped from 1 to 0.1 % the  $\text{NH}_3$  formations from the two nucleotides become the same. It is of interest to note how closely the deamination rates of the two substances approach each other in these different tissues. *From the composition of this second group we may infer that the general visceral deamination of adenine nucleotide is an indirect process.* The auricle of the heart is an interesting exception.

Table VI

I	II	III	IV	V	VI	VII	VIII
Tissue	Exp no	pH	Nucleotide concentration %	M. adenylic acid deaminated $\mu\text{g}$ N ml., mm. (1: dilution)	V. adenylic acid deaminated $\mu\text{g}$ N, ml., mm. (1: dilution)	True nucleotide deaminated at 0.1 % $\mu\text{g}$ N ml. min. (1: dilution)	Ratio of VI to V %
Voluntary muscle	7	7.0	1.0	987.0	0.05		0.005
	7	7.0	0.1	886.0	0.05	886.0	0.005
Cerebral cortex	4	7.0	1.0	17.1	2.7		15.8
	2	7.0	0.1	3.22	0.55	2.87	10.9
Sciatic nerve	4	7.0	1.0	5.4	0.70		13.0
	2	7.0	0.1	2.59	0.61	1.98	23.6
Auricle	4	7.0	1.0	13.3	2.70		20.3
	2	7.0	0.1	2.93	0.72	2.21	24.8
Blood	3	7.4	1.0	4.25	0.48		11.3
	3	7.4	0.05	0.45	0.04	0.41	8.9
Blood	6	7.0	1.0	12.2	0.60		4.9
	6	7.0	0.1	0.27	—	< 0.27	
Salivary gland	4	7.0	1.0	14.3	8.8		61.6
	2	7.0	0.1	3.18	3.05	0.03	95.8
Kidney	1	7.0	1.0	6.50	3.68		56.8
	1	7.0	0.1	1.67	1.61	0.06	96.6
Liver	1	7.0	1.0	5.76	4.04		70.2
	1	7.0	0.1	1.13	1.07	0.06	94.6
Stomach muscle	4	7.0	1.0	6.10	1.50		24.6
	2	7.0	0.1	0.18	0.19	– 0.01	105.5
Jejunum	4	7.0	1.0	4.70	2.30		48.9
	2	7.0	0.1	1.21	1.12	0.09	92.8
Ventricle of heart	1	7.0	1.0	4.72	4.78		101.5
			0.1			(0.00)	
Blood	5	8.3	0.1	0.028	0.027	0.01	96.4

The rabbit tissues were extracted with water, 1 part by weight to 20 vol. 1 vol. of extract was added to 1 vol. of nucleotide at pH 7.0. No other buffering was used except for blood and voluntary muscle, but the  $\text{NH}_3$  formation was not allowed to exceed  $N/5000$  strength, and no appreciable change of pH occurred. In the blood experiments 3, 6 and 5  $\text{CO}_2$ /bicarbonate ( $M/40$ ), maleic acid ( $M/20$ ) and bicarbonate ( $M/10$ ) were used, the blood dilution ranging from 1 in 5 to 1 in 8.8. Maleic acid buffering was used with the voluntary muscle extract. Room temperature.

For each experiment the tissues from two rabbits were used immediately after killing. After grinding the extract was usually allowed to stand for a few hours.



The above studies of deamination were carried out without further buffering than that provided by the nucleotide or the tissue extract. At the 1 % levels the nucleotide buffering is very appreciable. With the 0.1 % additions the  $\text{NH}_3$  formation was not allowed to exceed about  $N/5000$  with the result that no appreciable shift in  $p\text{H}$  occurred.

Seeing that in these 1 in 40 extracts the tissue  $\text{Mg}$  concentration has fallen considerably the effect of  $\text{Mg}$  addition was examined. No appreciable change from the above description was found.

#### THE DISTRIBUTION OF THE DEAMINASES IN TISSUES

These were measured by the deamination rates of adenosine and adenylic acid under standard conditions, and the unit chosen was the  $\mu\text{g. N/ml./min.}$ , the amount of enzyme being given per 1 g. or 1 ml. of the original tissue. A  $p\text{H}$  of 7.0 was selected as most suitable since it corresponds to the optimum formation of  $\text{NH}_3$  from adenylic acid in tissues, is most representative of the actual tissue  $p\text{H}$  and is also close to the adenosine optimum (multiplying the adenosine results by 1.09 will give the value for the optimum  $p\text{H}$ ).

1 in 20 water extracts of the tissues were made—grinding to a fine suspension with quartz sand—and an equal volume of substrate added. For adenylic acid

Table VII

Tissue	Adenosine deaminated	M. adenylic acid deaminated at 1 % (direct and indirect)	Tissue	Adenosine deaminated	M. adenylic acid deaminated at 1 % (direct and indirect)
<b>Alimentary:</b>			<b>Nervous:</b>		
Appendix	59.7 (2)	8.3 (2)	Spinal cord	14.7 (1)	14.8 (1)
Jejunum	52.6 (3)	6.6 (4)	Brain (whole)	8.6 (1)	12.2 (2)
Peyer's patches	36.8 (1)	12.1 (1)	Cerebral cortex	5.6 (1)	15.9 (3)
Duodenum	29.6 (1)	—	Pituitary	2.6 (2)	24.6 (2)
Duodenum } mucosa	23.1 (2)	14.4 (2)	Sciatic nerve	0.8 (3)	4.8 (3)
Jejunum } scrapings	21.1 (2)	11.6 (2)	<b>Respiratory:</b>		
Colon	10.7 (1)	—	Lungs	8.0 (3)	6.0 (2)
Ileum	8.1 (2)	—	<b>Circulatory:</b>		
Caecum	6.5 (1)	—	Auricle	6.8 (6)	9.4 (2)
Pyloric mucosa	2.8 (2)	2.2 (2)	Whole blood	5.9 (5)	14.0 (5)
Stomach muscle	2.0 (3)	5.1 (4)	Artery	4.1 (2)	2.2 (2)
<b>Glandular:</b>			Ventricle	2.3 (2)	2.8 (2)
Spleen	40.2 (3)	14.8 (2)	Plasma	0.04 (2)	0.04 (2)
Testicles	26.6 (3)	16.5 (2)	<b>Miscellaneous:</b>		
Salivary glands	19.7 (5)	9.8 (4)	Embryonic tissue	10.6 (2)	10.7 (2)
Suprarenals	15.3 (3)	15.5 (2)	Bone marrow	8.3 (2)	9.7 (2)
Pancreas	12.6 (3)	3.0 (2)	Uterus	3.7 (1)	6.5 (1)
Thyroid	8.3 (2)	—	Skin	0.0 (1)	0.0 (1)
Kidney	7.7 (2)	5.6 (2)	Bone	0.0 (1)	0.0 (1)
Ovary	6.5 (2)	5.6 (1)			
Liver	3.7 (3)	2.8 (3)			
Pituitary	2.6 (2)	24.6 (2)			
<b>Muscular:</b>					
Auricle	6.8 (6)	9.4 (2)			
Diaphragm	2.5 (2)	108.0 (2)			
Ventricle	2.3 (2)	2.8 (2)			
Stomach muscle	2.0 (3)	5.1 (4)			
Skeletal muscle	0.9 (4)	1145.0 (6)			

The units in which the deamination is expressed are  $\mu\text{g. N/ml./min.}$  for the original tissue.

Tissues ground with quartz in 20 vol. water. To 1 vol. extract 1 vol. of nucleoside plus 0.5 vol. buffer (phosphate  $M/20$ ) or 1 vol. extract plus 1 vol. of 2 % nucleotide.  $p\text{H}$  7.0. Room temperature.

this consisted of 2 % nucleotide which had been neutralized to pH 7.0 with NaOH and for adenosine 0.5 % adenosine buffered with phosphate at pH 7.0 to make *M*/20 in the mixture. No buffer except that of the nucleotide itself in 1 % strength in the mixture was used in studying the distribution of the adenylic deaminase.

The unexpected behaviour of adenylic deaminase in tissues as regards the points already considered led us to examine the efficiency of extraction of the enzyme under various conditions. In our experiments the efficiency of extraction is somewhat variable even under strictly standardized conditions, though this may be attributable to varying distribution throughout the tissue. Using pure water, 0.9 % NaCl and 0.9 % NaCl plus 0.05 % KCl we obtained a ratio of 1.0 : 1.2 : 1.6 in the amounts of enzyme action observed in a few experiments. These extractions were made from the same muscle finely cut and mixed, some of which was weighed out and ground with quartz and the fluid to make 1 in 20 extracts. The inclusion of Mg and Ca with the 0.9 % NaCl caused a decrease, which also resulted from the inclusion of some bicarbonate in the extract medium. From this it will appear that saline would have been a more efficient extractor than water and there is also a case for supposing K addition to act better than Na alone, though to decide this many more experiments would be necessary. Since for reasons already given the amount of the deaminase can be given only in a comparative way, the water extract was considered sufficiently serviceable.

Table VII gives a summary of the experiments performed on 36 tissues of the rabbit, the results being considered in the subsequent discussion. The adenylic deamination given in Table VII indicates the sum of both direct and indirect formations at 1 % concentration, from which, as already considered, we must distinguish the pure nucleotide deamination and the effective deamination rate in tissues.

#### DISCUSSION

*The distribution of adenylic acid and adenosine deaminases in the tissues of the rabbit.* The distribution of the adenosine deaminase may be roughly divided into a digestive, a glandular, a nervous and a muscular type and in that order with respect to enzyme strength. The jejunum and duodenum were found to contain 53 and 30 units respectively while the appendix showed the highest of any tissue examined, namely 60 units. In each case all the intestinal coats were taken together, but scrapings from the mucosa of the duodenum and jejunum gave also no increase over these figures, but rather a decrease.

An explanation of the high appendix concentration compared with that in the ileum, caecum and colon may be given by considering that in the rabbit—and possibly in other herbivora—the caecum serves as a second stomach in which the cellulose walls of cells are digested by special bacteria, further nucleic acid material being liberated. The appendix may then be represented as playing the same role as the duodenum and jejunum to the main organ with respect to the further treatment of nucleic acid.

In the glandular distribution the spleen comes highest, but this tissue is exceptional with regard to the adenylypyrophosphate liberated therein from the red corpuscles. The special glandular activity is shown rather by the testicles, salivary glands, suprarenal and the pancreas, which gave from 27 to 13 units. Of the eleven types of glandular tissue examined the liver gave the second lowest with 4 units and the pituitary lowest with 3 (the pituitary contained relatively a very high proportion of adenylic acid deaminase, namely 25 units).

The nervous distribution of adenosine deaminase is somewhat similar to the glandular, being perhaps a little lower. The difference here is in the compara-

tively higher amount of adenylic deaminase, the ratio being about 3 to 1 in the cerebral cortex and 6 to 1 in the conducting nerve (the latter containing only 0.8 units of adenosine deaminase).

Muscle tissue in general contained the lowest amounts, with the auricle of the heart highest in the group, having 6.8 units, and voluntary muscle lowest with 0.9. The muscle of the stomach wall had 2.0 units and the ventricle of the heart 2.3.

Concerning the distribution of true adenylic acid deaminase, voluntary muscle is very exceptional with an average of 1145 units. A long way after this we have nervous tissue, e.g. cerebral cortex, with 15.8 units (vide Table VI) but only an effective deamination rate of less than 2.9. Conducting nerve also has a high ratio of adenylic acid deaminase to that of adenosine (6:1). Blood was found to contain 14 units and the auricle of the heart 9–11. Apart from these tissues the amount of true adenylic acid deaminase found elsewhere was low and its effective action practically zero. Contrary to our first observations plasma was found to contain a very small and variable amount of the true nucleotide deaminase, but at any possible normal concentrations its activity is only a few % of the adenosine deaminase therein (0.05 unit).

#### *The significance of the adenylic acid and adenosine deamination*

That  $\text{NH}_3$  formation is coincident with activity in tissues is a point that, as is well known, has been emphasized by Embden and others. Adenylic acid was shown by Embden to be the precursor of this  $\text{NH}_3$  in voluntary muscle. Later it was found by Lohmann [1929] that adenylic acid was itself a stage in the breakdown of adenylypyrophosphate, the role of which in phosphate transference and the glycolytic cycle was soon recognized. It was subsequently considered—in particular by Parnas—that the deamination of the adenylic acid formed on dephosphorylation and such as escaped resynthesis was merely a detoxication process without any special relation to muscular activity. Certain findings in the present paper point directly against this view and may be summarized as follows.

- (1) An average of about 40 times more true adenylic acid deaminase exists in voluntary muscle than in any other tissue examined, but the effective deamination rate is greater by about 500–1000 times or more, owing to the absence therefrom—or at least the ineffective action—of specific inhibitors of the enzyme.

- (2) Though quite considerable amounts of the deaminase exist in the red corpuscle and other tissues, the action of the enzyme is reduced to a few % or less of its full action by special inhibition (in addition to that produced by the  $\text{CO}_2$  and bicarbonate system). In this way adenylic acid can be said to be conserved and presumably as a physiological process. Yet adenylic acid is freely permeable across the membrane of the red corpuscle and will therefore escape into the plasma. We have demonstrated this permeability to adenylic acid added both to oxalated and to heparinized blood and examined immediately after shedding, and also a similar permeability for voluntary muscle.

- (3) An adenine nucleotide apparently already exists in plasma and its pharmacological action cannot be considered as markedly less than that of muscle adenylic acid [Bennet & Drury, 1931].

- (4) The deamination of adenine nucleotide in the duodenum or jejunum is not direct but occurs after dephosphorylation. Here the total effective deamination of the nucleotide for normal concentrations is only a minute fraction of that in skeletal muscle—probably considerably less than 1 %—yet we may suppose that across the intestinal wall there pass amounts of nucleotide at least as great

as the adenylic acid set free momentarily and escaping re-esterification in voluntary muscle.

A point which has appeared conclusive to Lohmann [1935] against the special role of adenylic deaminase is the absence—as he found—of this enzyme in the muscle of the crab—including the claw muscle. We have investigated this point in turn and found for the claw muscles from four crabs a mean value of 40 units of the deaminase. While this amounts only to about 4 % of that in the rabbit's leg muscle, it is still very appreciable and higher than that found in any of the other tissues of the rabbit. Considering the many factors discussed above which inhibit the action of this enzyme it is not surprising that its absence is occasionally and erroneously concluded.

Direct nucleotide deamination can also occur normally in nerve tissue, the auricle of the heart and in red corpuscles though these have no obvious relationship. It may be noted that the conservation of adenylic acid in general tissues by the action of the specific inhibitors and  $\text{CO}_2$  (or bicarbonate) combined with its permeability (as we may deduce from muscle and red corpuscles) may lead to the activity of this substance as a local vasodilator.

Concerning the deamination of adenosine, this may be firstly considered as merely a process in the metabolism of surplus nucleic acid, and in the intestinal wall all the normal deamination of adenine nucleotide derives from this substance. Besides this, the widespread occurrence of adenosine deaminase in tissues and in blood may be regarded from two aspects. Adenosine may be looked upon as an accidental formation from free adenylic acid, set free in turn from A.T.P. in the glycolytic process, and that when formed it is detoxicated by deamination. Adenosine is apparently the most active substance pharmacologically of its class, and that it should be rapidly deaminated in the intestinal wall may be linked with the detoxication view. We may consider too that adenosine deamination in the general tissues is a physiological mechanism for obtaining a ready supply of  $\text{NH}_3$ —which they cannot obtain directly from blood --and that the role of this  $\text{NH}_3$  may be of a similar kind, though much more slowly formed, than the  $\text{NH}_3$  in voluntary muscle. What this role may be is so far obscure, but various possibilities can be suggested such as permeability changes caused thereby, requirement for local synthesis of amino compounds, local neutralizations etc.

#### SUMMARY

1. The relation of adenylic acid deaminase to substrate concentration is affected by specific buffer inhibition and also by special inhibitors in tissues. These appear to act by displacing the adenylic acid from an adsorbing surface. Among the buffers so acting are  $\text{CO}_2$  and bicarbonate, phosphate and veronal. Maleic and citric acids do not act in this way. The effect of the inhibition is to simulate a linear relation of action to substrate up to very high concentrations (about 1 % or more). The special tissue inhibitors are either absent from or ineffective in voluntary muscle. Dilution of blood—by lessening the concentration of the inhibitors can very greatly increase the deamination of adenylic acid per ml. blood.

Similar inhibitions of the action of adenosine deaminase have not been found.

2. Methods for determining the path of the deamination of adenine nucleotide are given. It is shown that (apart from voluntary muscle) nerve tissue, the auricular muscle of the heart and red corpuscles deaminate adenylic acid normally in a direct way, but the kidney, liver, intestine, smooth muscle, ventricular muscle of heart and blood after loss of  $\text{CO}_2$  deaminate it only after an initial dephosphorylation.

3. The distribution of adenosine and adenylic acid deaminases has been studied for 36 tissues of the rabbit. The amount of adenylic acid deaminase in voluntary muscle has a mean value 40 times greater than that in any other tissue examined, but its effective action on possible normal concentrations is greater by about 500–1000 times or more. Through the inhibition of the adenylic acid deaminase in blood, although there is more of this than the corresponding adenosine enzyme, the action on minute amounts is less than 1 % of that on the nucleoside. Even those tissues, apart from voluntary muscle, which normally deaminate the nucleotide directly are less effective deaminators of small amounts of adenylic acid than of adenosine.

The highest concentration of adenosine deaminase was found in the vermiform appendix and somewhat lesser amounts in the duodenum and jejunum.

4. Contrary to Lohmann's finding [1935], adenylic acid deaminase in very appreciable amounts was found in the claw muscles of the crab.

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# LX. THE INFLUENCE OF LOW AND HIGH PLANES OF NUTRITION ON THE COMPOSITION AND SYNTHESIS OF FAT IN THE PIG

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THE work described in this communication deals with the determination of the component acids in the depot fats of pigs which had received controlled diets at different levels of nutrition. The primary object was to ascertain how far the quantitative proportions of the various component acids varied in relation to the feeding level of the animal. It was also found possible to make a rough quantitative comparison of the amounts of each fatty acid (*a*) ingested by the animal, (*b*) deposited in the animal in the form of fat, and therefrom to deduce the amount and nature of fat synthesized in the animal, and also to compare the rates of deposition of fat in animals which had been given different rations.

An investigation has recently been in progress at the Animal Nutrition Institute of the School of Agriculture, Cambridge, designed to determine the effect of rate of growth on the proportions of the different parts and tissues in the pig. The physiological and anatomical results of this investigation are in course of publication by Dr C. P. McMeekan. By the courtesy of Dr J. Hammond, specimens of the back and perinephric fats, in sufficient amounts for detailed component acid analyses to be made, were placed at our disposal, together with details of the diets received by the pigs and of the weights of their fatty tissues at the time of slaughter. We are informed by Dr Hammond that the plan of feeding was as follows.

Thirty pigs of unusually homogeneous (inbred) stock were fed on the same diet in different amounts. Up to the age of 16 weeks the pigs were in two groups:

- (*a*) On high plane of nutrition, final weight 120–132 lb. (average 126 lb.).
- (*b*) On low plane of nutrition, final weight 32–37 lb. (average 34 lb.).

Subsequently half of the pigs in each group continued to receive food at the same plane as during the first 16 weeks, whilst the other half were transferred to the alternative level, those on the high plane now receiving the low plane diet, and conversely. The restriction of the ration was that required to make the pigs grow according to pre-arranged growth curves. All these animals were grown to 200 lb. weight before slaughtering.

Details of the rations supplied to the animals were as follows:

	High plane	Low plane
Ration while on sow ...	Free access to sow and to dry meal mixture no. 1; separated milk <i>ad lib.</i>	Restricted access to sow and to dry meal mixture no. 1 and to separated milk
Ration from weaning to 16 weeks ...	1 gal. separated milk per pig daily and meal mixture no. 1 <i>ad lib.</i>	$\frac{1}{2}$ gal. separated milk per pig daily and restricted ration meal mixture no. 1
Ration from 16 weeks onwards ...	1 gal. separated milk per pig daily and meal mixture no. 2 <i>ad lib.</i>	$\frac{1}{2}$ gal. separated milk per pig daily and restricted ration meal mixture no. 2

Composition of meal mixtures

No. 1	%	No. 2	%
Dried separated milk	20	Barley meal	30
White fish meal	30	White fish meal	30
Wheat middlings	30	Flaked meal	30
Flaked meal	20	Wheat middlings	10

The first group of pigs had passed the 16 weeks period before it was decided to reserve sufficient amounts of the back and perinephric fats for the present work, but these specimens were obtained from the final animals killed at 200 lb. weight (pigs nos. 72, 73, 74, 82); a duplicate experiment up to 16 weeks was carried out later from which samples of fat (pigs nos. 138 and 139) were supplied for our analyses.

The weights of fat deposited in the various tissues of each of the animals are summarized in Table I from data kindly supplied to us by Dr C. P. McMeekan (working with Dr Hammond).

Table I. *Total weight, etc., of fat in tissues*

Pig ...	73 (hog)	74 (gilt)	72 (hog)	82 (gilt)	138 (gilt)	139 (gilt)
Planes of diet ...	Low-High	High-High	High-Low	Low-Low	High (16 weeks)	Low (16 weeks)
Age (days) ...	212	181	212	339	112	112
Farm weight (lb.) ...	204	198	198	197	132	37
Fat in tissues (kg.):						
Subcutaneous	21.69	16.46	15.41	9.80	8.73	0.31
Perinephric and kidney	2.00	1.46	1.50	1.04	0.74	0.02
Intermuscular	6.20	5.96	4.68	4.33	3.27	0.16
Mesenteric	0.95	1.08	0.79	0.93	0.57	0.05
Caul	0.24	0.15	0.11	0.12	0.07	—
Total fat (kg.)	31.08	25.11	22.40	16.22	13.38	0.54
Rate of deposition of fat (g. per diem):						
Subcutaneous	102	91	73	20	78	3
Perinephric	9	8	7	3	7	0.2
Total body fat	147	139	106	48	119	5

Apart from insufficiency of material in certain instances, it was out of the question to carry out detailed fatty acid analyses on each of the 34 depot fats in Table I. Large specimens (1000–1200 g.) of the outer and inner back fats, and of the perinephric fats, were however prepared by extracting the fatty tissues with acetone [cf. Banks & Hilditch, 1932]. After a preliminary analysis of both inner and outer back fats<sup>1</sup> from pig no. 74, which showed differences between the two fats similar to those previously noted [Banks & Hilditch, 1932; Dean & Hilditch, 1933, 2], it was decided to confine detailed examination for the time

<sup>1</sup> I.e. the layers of fat on the inner and outer side of the well-marked dividing line of connective tissue in the subcutaneous fatty layer lying on the thorax and loin of the pig.

being to the outer back fats. This choice was mainly determined by the fact that the unsaturated acids (in which we were particularly interested from the standpoint of keeping qualities of the fat) were known to be present in greater proportions in this section of the fats than in the inner back fat or the perinephric fat. The general characteristics of all the fats which were extracted in quantity are, however, given in Table II.

Table II

Pig no.	Planes of diet	Peri-nephric fat	Inner back fat	Outer back fat		
		i.v.	i.v.	i.v.	Sap. equiv.	Unsap (%)
73 (hog)	Low-High	52.4	55.6	58.5	288.8	0.2
74 (gilt)	High-High	49.5	54.4*	59.8	285.9	0.2
72 (hog)	High-Low	53.3	57.2	63.3	288.0	0.1
82 (gilt)	Low-Low	57.4	60.4	65.5	286.5	0.1
138 (gilt)	High (16 weeks)	51.0	56.0	60.5	†	†
139 (gilt)	Low (16 weeks)	-	†	†	†	†

\* Sap. equiv. 285.4, unsaponifiable 0.1%.

† No. 138, composite back fat, i.v. 58.0, sap. equiv. 284.9, unsaponifiable 0.1%.

‡ No. 139, composite back fat, i.v. 62.9, sap. equiv. 287.8, unsaponifiable 0.2%.

### EXPERIMENTAL

Determination of component fatty acids followed the lines which have been described in recent communications from this laboratory: (a) separation of the mixed fatty acids from about 200 g. of each fat into "solid" and "liquid" acids by the different solubilities of the lead salts in alcohol, and (b) fractional distillation at low pressure of the methyl esters of each group of fatty acids. As a preliminary to the examination of a typical depot fat from each of the six experimental animals, both the inner and outer back fats of the pig which had been fed throughout on the "high" plane of nutrition were studied.

In the case of the inner back fat, separate portions of the esters of the "liquid" acids were distilled by the simple "Willstätter bulb" apparatus, and through an electrically heated and packed column which gives sharper separation of some of the minor components [Longenecker, 1937; Hilditch & Longenecker, 1937]. This showed that the data obtained with the simpler fractionation apparatus, when calculated in terms of all the acids shown to be present by use of the more elaborate column, give results which accord with those from the latter, when dealing with the comparatively simple mixture of fatty acids present in pig and similar depot fats (Table III). Nevertheless the electrically heated column, although it involves a longer period of distillation and also more practice in operation in order to obtain adequate control, gives extremely sharp separation of the successive ester fractions. In the main part of the work the esters of "solid" acids were distilled from a Willstätter bulb, and those of the "liquid" acids through the electrically heated column.

The proportions of the component acids of the inner (Table III) and outer (Table IV) back fats of pig no. 74 have the same relationship to each other as in previously examined specimens of inner and outer back fats from individual pigs [Banks & Hilditch, 1932; Dean & Hilditch, 1933, 2]. The minor component acids are not significantly different, the percentage of palmitic acid is slightly lower in the outer than in the inner back fat, and the chief difference is an increase in the oleic acid content of the outer fat, mainly at the expense of stearic acid.



Table III. *Component acids of inner back fat of pig no. 74*

Fractionation apparatus ...	"Solid" acids 44.8%	"Liquid" acids 55.2%		Total fatty acids (excluding unsaponifiable matter)			
				% (wt.)		% (mol.)	
		W.	E.H.P.	W.	E.H.P.	W.	E.H.P.
Myristic acid	0.03	0.47	0.99	0.5	1.0	0.6	1.2
Palmitic acid	27.41	3.62	2.73	31.1	30.1	33.1	32.2
Stearic acid	16.14	—	—	16.2	16.2	15.5	15.5
Tetradecenoic acid	—	—	0.28	—	0.3	—	0.3
Hexadecenoic acid	—	2.88	2.69	2.9	2.7	3.1	2.9
Oleic acid	1.19	39.73	39.65	41.0	40.9	39.7	39.6
Linoleic acid	—	7.10	7.10	7.1	7.1	6.9	6.9
Unsaturated C <sub>20-22</sub> acids	—	1.27	1.67	1.3	1.7	1.1	1.4
Unsaponifiable	0.03	0.13	0.09	—	—	—	—

W. = esters distilled from a Willstätter bulb.

E.H.P. = "liquid" esters distilled through the electrically heated and packed column.

*Constitution of the hexadecenoic acid of pig depot fats.* Hilditch & Shorland [1937] found evidence, contrary to previous observations, of the presence of perhaps 3–4% of hexadecenoic acid in pig depot fats. The present series of analyses show that pigs of about 200 lb. live weight contain 2–3% of this acid in the mixed acids of the back fats, and the opportunity was taken to ascertain its structure.

A number of the ester fractions, with equivalents ranging from 260 to 280 and I.V. from 40 to 70, obtained from methyl esters of the "liquid" acids of the pig back fats, were united, and their mixed acids submitted to the lead salt-alcohol separation to remove as much palmitic acid as possible. After reconversion of the "liquid" acids thus obtained into methyl esters (28.4 g.), a further distillation through the electrically heated column gave a fraction (11.6 g., sap. equiv. 268.0, I.V. 86.7, CNS value 87.1) which appeared to be substantially methyl hexadecenoate (sap. equiv. 268.0, I.V. 94.8) with minor proportions of methyl palmitate and, perhaps, myristate. (It will be noted that methyl  $\Delta^{9:10}$ -hexadecenoate reacts normally as a monoethenoid derivative with Kaufmann's thiocyanogen reagent, the thiocyanogen and iodine values being identical.)

Oxidation of this ester in acetone solution with powdered  $\text{KMnO}_4$  gave azelaic acid (M.P. and mixed M.P. 101°) and heptanoic acid (Et ester, sap. equiv.: found 154.3, calc. 158.0). The acid in pig depot fat, like that of ox depot fat [Hilditch & Longenecker, 1937], is therefore  $\Delta^{9:10}$ -hexadecenoic acid. This was further confirmed by the production, on oxidation of a dilute alkaline solution of the acid with  $\text{KMnO}_4$ , of 9:10-dihydroxypalmitic acid (M.P. and mixed M.P., 125°).

*Nature of the highly unsaturated C<sub>20-22</sub> acids present in pig depot fats.* Brown & Deck [1930] showed that pig fats contained 0.3–0.4% of highly unsaturated acids, estimated as arachidonic acid,  $\text{C}_{20}\text{H}_{32}\text{O}_2$ , from the yield of polybromides from the distilled esters of the lard fatty acids (employing the observed yield of polybromides from methyl arachidonate, 77.6<sup>1</sup>). The approximate proportion of C<sub>20-22</sub> acids, estimated from the equivalents of the highest-boiling fractions of the methyl esters, was found to be somewhat larger (ca. 1%), by Banks & Hilditch [1932] and Dean & Hilditch [1933, 2], whilst in the present series it was uniformly found to be about 2%.

<sup>1</sup> Subsequently Ault & Brown [1934] gave the yield of insoluble polybromo-adducts from pure methyl arachidonate as 86.5% of the weight of the ester.

In the present experiments, the polybromo-adducts from accumulated high-boiling fractions from the pig back fats were compared with those from the oil extracted from the fish meal used in the diet of the animals. The polybromo-additive products from each source were debrominated in pyridine solution with zinc [Kaufmann & Mestern, 1936]; the respective products had the following characteristics:

	Acids from debrominated pig fat polybromo- adducts	Acids from debrominated fish meal oil polybromo- adducts
Appearance	Pasty solid	Liquid
Yield from polybromo-adducts	33.8%	27.6%
I.V.	314	336
CNS value	94.5	178

These figures support Brown's observation [1931] that the deposited  $C_{20-22}$  acids differ from those ingested as fish oil, and are less unsaturated than the latter; but they do not necessarily imply that arachidonic acid is the only acid concerned. The equivalents of the residual esters left after fractionating the esters of the "liquid" acids (after correcting for any unsaponifiable matter present) were never below that of the ester of a  $C_{20}$  acid, and in one case the figure was considerably higher. Since these residual esters must in all cases have still contained some oleate, it follows that some quantity of esters of  $C_{22}$  acids was also present.

*Component fatty acids of the outer back fats of pigs killed at 200 lb. live weight*

About 300 g. of the outer layer of the back fat of each of the four animals fattened to 200 lb. live weight at different planes of nutrition were converted into the mixed fatty acids, which were then resolved into "solid" and "liquid" acids as described earlier. The methyl esters of the "solid" acids were fractionated from a Willstätter bulb, and those of the "liquid" acids through the electrically heated column. The lengthy detailed fractionation data are unnecessary for the subsequent discussion, and only the final results of the analyses need be recorded here. In Table IV the proportions of the component acids are given in the form of both weight and molar percentages.

Table IV. *Component acids of outer back fats (pigs killed at 200 lb. wt.)*

Pig no. ...	73		74		72		82	
Sex ...	Hog		Gilt		Hog		Gilt	
Planes of nutrition*	Low-High		High-High		High-Low		Low-Low	
	% wt.	% mol.	% wt.	% mol.	% wt.	% mol.	% wt.	% mol.
Myristic acid	1.1†	1.4†	1.3	1.6	0.7†	0.8†	0.8†	1.1†
Palmitic acid	28.2	30.2	28.3	30.2	25.3	27.2	25.9	27.8
Stearic acid	13.5	13.0	11.9	11.5	13.1	12.7	12.2	11.8
Tetradecenoic acid	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2
Hexadecenoic acid	2.4	2.6	2.7	2.9	2.0	2.1	2.0	2.1
Oleic acid	47.0	45.4	47.5	46.0	51.0	49.7	48.1	46.8
Linoleic acid	5.2	5.1	6.0	5.8	5.3	5.2	7.8	7.6
Unsaturated $C_{20-22}$ acids	2.4	2.1	2.1	1.8	2.5	2.2	3.0	2.6

\* I.e. up to age of 16 weeks, and from 16 weeks to slaughter.

† Includes traces of a lower saturated (lauric?) acid.

*Component fatty acids of composite back fats  
of pigs killed at age of 16 weeks*

The determinations were carried out as in the preceding cases, but only about 40 g. were available of the total back fat of the pig fed on the low plane of nutrition. For this reason a specimen of the composite (inner+outer) back fat of each animal was examined in these cases. The final results are given in Table V.

Table V. *Component acids of composite back fats (pigs 16 weeks old)*

Pig no.	...	...	138	139
Sex	...	...	Gilt	Gilt
Plane of nutrition	...	...	High	Low
	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Myristic acid	1.0*	1.2*	1.3	1.5
Palmitic acid	29.8	31.7	28.1	29.9
Stearic acid	12.7	12.2	11.8	11.3
Tetradecenoic acid	0.2	0.2	Trace	Trace
Hexadecenoic acid	3.5	3.8	4.8	5.2
Oleic acid	47.8	46.2	42.9	41.5
Linoleic acid	3.1	3.1	8.2	8.0
Unsaturated C <sub>20-22</sub> acids	1.9	1.6	2.9	2.6

\* Includes traces of a lower saturated (lauric?) acid.

### DISCUSSION

It is well recognized that diets rich in fat cause pigs to deposit much of the ingested fat and that, for example, the inclusion of liquid fats rich in oleic and linoleic glycerides, such as cottonseed, maize or groundnut oils, in the ration tends towards the production of soft and oily pig fats [cf. Ellis *et al.* 1926, 1, 2; 1931: 1938]. In the present experiments we are less concerned with the problem of ingested fat than of that synthesized by the animal, since the total fat content of the diets was very low. The fat content of the meal mixtures was 2.2%, whilst that of the separated milk (which formed the major constituent of all the feeds) was of the order of 0.1%; the sow's milk taken before weaning contained about 4.5% of fat. The percentage of fat in the whole of the food taken by the pigs varied somewhat according to the different ratios of separated milk and meals taken in the various experiments, but it never exceeded 1% of the total diet, the extreme limits being 0.5% at the "low-low" and 1.0% at the "high-high" planes of nutrition. Any significant changes in the composition of the depot fats must therefore result almost wholly from differences in the amount of food given to the animal, and not from any sensible alteration in the amount of fat ingested.

We may take in the first place differences in the proportions of the various component acids (Tables IV and V), although these are less notable in percentage form than when the total amount of each component deposited as fat by the animal is considered (cf. below, Table VII). In the group of outer back fats, there is a slight but apparently definite increase in oleic acid content in the case of the 200 lb. weight animals which were fed from 16 weeks onwards on a restricted diet. Parallel with this, the palmitic acid contents in these instances are somewhat lower than in the outer back fats of pigs fattened on the higher plane of nutrition; and this also holds for the feeding at the two dietary levels during the first 16 weeks. In all cases, the variation is within the limits of

$30 \pm 3\%$  (mol.) which have been found to be the characteristic palmitic acid content of the acids of herbivorous animal depot fats [Hilditch & Longenecker, 1937]. The stearic acid contents of all six outer back fats showed little variation.

Of the minor components, it will be seen that only traces of saturated acids of lower molecular weight than myristic acid are present. Hexadecenoic (and traces of tetradecenoic) acids are fairly constant at 2.0–2.7% in the fats of the 200 lb. weight animals, but somewhat higher at the 16 weeks' stage, irrespective of the plane of nutrition. The unsaturated  $C_{20-22}$  acids are more prominent than has been observed in previous cases; and in this connexion it should be noted that the proportion of fish meal, 10–13% of the total diets, was fairly considerable. Woodman *et al.* [1937] state that inclusion of 5% of fish meal in the ration up to the time of slaughtering involves a risk of producing a fishy taint in bacon.

The most variable of the minor constituents is linoleic acid. In fats from 200 lb. weight animals fattened on the high plane diet, this formed about 5–6% of the fatty acids, whilst at 16 weeks (high plane) it was only 3%; but it reached 8% of the total outer back fatty acids of the animals fed on the restricted ration, either at 16 weeks or throughout until the pig had reached 200 lb. live weight. This variation is significant in regard to the relative softness of the depot fats (and, possibly, their keeping quality; although in the latter respect other factors may also come into play). Differences in linoleic acid content, though small numerically, can have an important effect on the glyceride structure of the fat [Green & Hilditch, 1938]: linoleic acid, as a minor component, will not be present more than once in any triglyceride molecule, whereas oleic acid, the most prominent component, will be present in the majority of the triglyceride molecules. Hence any linoleic acid is almost certain to be present as a linoleo-oleo-glyceride, and of course glycerides containing two unsaturated acyl groups are liquid at the ordinary temperature. Consequently, whilst a content of 5% of linoleic acid connotes about 15% of corresponding liquid glycerides, 8% of linoleic acid involves the presence of about 24% of liquid glycerides—nearly 10% more of the fat being of a type of lower melting point in consequence of an increase of 3% in the linoleic acid percentage. The increases observed, at low planes of nutrition, in the linoleic, and also the oleic, acid contents of the depot fats are clearly the cause of the already known fact [Ellis *et al.* 1925; 1930; Robison, 1931; Callow, 1937] that rapid growth of pigs (i.e. increased rate of fat deposition) leads to the production of firmer fat.

Apart from increased tendency to softness of fat, the uneconomic rate of production of fat at low as compared with higher planes of nutrition is clearly evident from the data in Table I.

The variations in the rate of fat deposition, and also in the mean unsaturation of the fats (so far as can be judged (Table II) from i.v. alone), are parallel in all the depots of the animal. The most rapid rates of production, and more saturated (i.e. firmer) fats, result when the diet after the first 16 weeks is on a high plane of nutrition. Abundant diet is more important during these latter stages of growth than during the first 16 weeks of life. As a matter of fact, the most rapid production, but not quite the firmest type, of fat resulted from restricted diet up to 16 weeks, followed by the high plane of nutrition for the remainder of the life of the animal. A "low plane" diet up to 16 weeks followed by "high plane", again, gives much better results both as regards rate of fat production and relative saturation of the fat than the alternative of "high plane" diet up to 16 weeks and then a restricted diet to 200 lb. weight.

It should be noted that these conclusions refer only to fat formation in the animal, and take no account of bone and muscle production. Thus, although the fat is softer and produced more slowly in the animal fed on the "high-low" planes of nutrition than in that fed on the "low-high" planes, the former animal is of more desirable conformation as a whole—long and lean—than the latter which is of the short, fat type.

The effects of restricted rations throughout the whole life of the pig are seen to be very uneconomical in the long period taken to reach 200 lb. live weight, the excessive consumption of food required to produce a given increase in body weight, and in the relatively unsaturated and soft character of the resulting depot fats.

The component acid data discussed above may be utilized to yield some information of an approximate order as to the amount of each individual fatty acid deposited by the pig, and to compare these figures with the corresponding amounts of the fatty acids consumed by each animal in its diet. Obviously, a number of approximations and estimates have to be made in order to obtain all the necessary data, but we have satisfied ourselves that any errors thereby introduced are insufficient to alter the general conclusions to be drawn later from this comparison. The computation of individual acids in the fats of the diet is that most liable to errors in estimation, but the total quantity of fat ingested by any animal is so much less than that deposited in the animal, that wider variations from our estimates of the component acids present than are at all likely would have only minor effects on the ultimate comparison between ingested and deposited fats. The following methods have been used in these calculations.

*Component acids ingested as fat in the diet.* The amount of each individual fatty acid combined as fat in 100 parts of each of the constituents of the food given to the pigs has been calculated with the results shown in Table VI. The component acid data used for the dietary fats have been taken from the following sources:

*Sow's milk fat.* The figures are from a partial analysis (not using ester-fractionation) [Laxa, 1931].

*Cow's milk fat.* The data obtained by ester-fractionation for a typical cow's milk fat of similar i.v. by Dean & Hilditch [1933, 1] have been used, but modified to include the 5% of lower unsaturated acids now known to be present [Hilditch & Thompson, 1936; Longenecker & Hilditch, 1938].

*White fish meal oil.* The component acids are taken from analyses by Lovern [1937] for halibut and turbot flesh fats (no others except herring being available). If the  $C_{20-22}$  acid contents in other fish flesh fats should be somewhat lower, such difference will not affect the conclusions drawn from Table VII (below).

*Wheatmeal, maize and barley oils.* The fatty acid data are based on recent determinations by modern methods respectively by Sullivan & Bailey [1936], Baughman & Jamieson [1921], and Täufel & Rusch [1929]. The i.v. of the meal oil differed somewhat from those of the oils analysed by these workers, i.e. the proportions of oleic and linoleic acids are not exactly those given. It is hardly legitimate, in the case of these seed fats, to adjust the proportions of these acids according to the i.v. of the fats (*vide infra*), but here again alternative calculations have shown that the differences thereby introduced have no significance in regard to the final comparison. In accordance with recent observations [Hilditch & Jasperson, 1938], however, 1% of hexadecenoic acid has been allowed in each case.

*Component acids deposited as fat in the pigs.* The data in each case depend upon our determinations of the acids in the outer back fats. Since it is now well

Table VI. *Approximate amounts of fatty acids present as fat in the dietary constituents*

Fatty acids	Sow's milk %	Separated milk %	Meal mixtures	
			No. 1 %	No. 2 %
Saturated:				
Below C <sub>14</sub>	0.04	0.011	0.02	—
Myristic	0.13	0.010	0.05	0.03
Palmitic	1.21	0.024	0.27	0.22
Stearic	—	0.007	0.06	0.05
Unsaturated:				
C <sub>18</sub> (and C <sub>14</sub> )	—	0.005	0.06	0.05
Oleic	2.94	0.034	0.58	0.63
Linoleic (and linolenic)	—	0.004	0.79	0.89
C <sub>20-22</sub>	—	—	0.24	0.24
	4.32	0.095	2.07	2.11

established that, within the fats of a single animal, or indeed over wider ranges of pig, ox and sheep depot fats, difference in total unsaturation is very largely conditioned by respective differences in the contents of oleic and stearic acids [cf. Tables III and IV: Banks & Hilditch, 1931; 1932; Dean & Hilditch, 1933, 2], it is quite safe in these cases, for small differences in i.v., to adjust the stearic and oleic acid contents determined on one of the depot fats of the animal concerned. This has been done in the case of each pig in the present series. The i.v. of the inner and outer back fats and the perinephric fats are given in Table II; the intermuscular fats were assumed to have similar i.v. to the inner back fats, and the mesenteric and caul fats to have similar i.v. to the perinephric fats. From the total weights of fat in each tissue (Table I) the individual acids deposited as fat were then calculated.

Table VII shows the weights (kg.) of each individual acid or group of acids thus calculated to have been (a) received as fat in the food or (b) deposited as fat

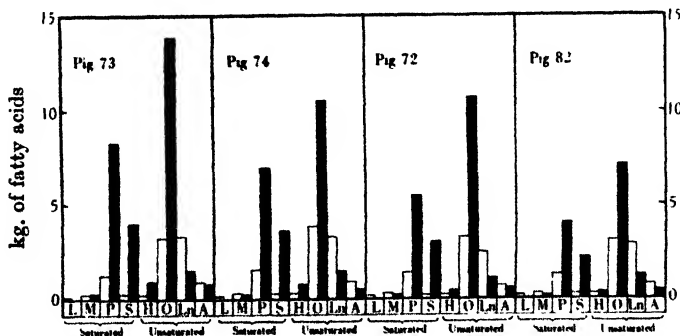


Fig. 1. Ingestion and deposition of fatty acids by pigs. Fatty acids: ingested, white; deposited in tissues, black. L, lauric (and lower saturated) acids; M, myristic acid; P, palmitic acid; S, stearic acid; H, hexadecenoic acid; O, oleic acid; Ln, linoleic acid; A, unsaturated C<sub>20-22</sub> acids.

in the tissues of the four experimental animals fattened to 200 lb. live weight and of the two animals killed at the age of 16 weeks. Fig. 1 is a graphical presentation of the same data.

Table VII. *Comparison of acids in fat ingested and deposited by pigs*

(a) Pigs at different planes of nutrition to 200 lb. wt.

Pig ... ..	73 (Hog)			74 (Gilt)		
Planes of nutrition ...	Low-High			High-High		
Fatty acids (kg.)	Fed	Deposited	Difference	Fed	Deposited	Difference
Saturated:						
Below C <sub>14</sub>	0.11	Trace	- 0.11	0.11	—	- 0.11
Myristic	0.24	0.29	+ 0.05	0.26	0.24	- 0.02
Palmitic	1.28	8.27	+ 6.99	1.53	6.92	+ 5.39
Stearic	0.25	3.99	+ 3.74	0.24	3.58	+ 3.34
Unsaturated:						
C <sub>16</sub> (and C <sub>14</sub> )	0.22	0.89	+ 0.67	0.22	0.72	+ 0.50
Oleic	3.24	13.87	+ 10.63	3.80	10.50	+ 6.70
Linoleic	3.30	1.48	- 1.82	3.22	1.43	- 1.79
C <sub>20-22</sub>	0.87	0.74	- 0.13	0.86	0.48	- 0.38
	9.51	29.53	+ 20.02	10.24	23.87	+ 13.63
Pig ... ..	72 (Hog)			82 (Gilt)		
Planes of nutrition ...	High-Low			Low-Low		
Fatty acids (kg.)	Fed	Deposited	Difference	Fed	Deposited	Difference
Saturated:						
Below C <sub>14</sub>	0.12	Trace	- 0.12	0.16	Trace	- 0.16
Myristic	0.24	0.21	- 0.03	0.27	0.15	- 0.12
Palmitic	1.36	5.45	+ 4.09	1.28	4.00	+ 2.72
Stearic	0.20	2.99	+ 2.79	0.25	2.16	+ 1.91
Unsaturated:						
C <sub>16</sub> (and C <sub>14</sub> )	0.17	0.43	+ 0.26	0.22	0.31	+ 0.09
Oleic	3.27	10.69	+ 7.42	3.07	7.09	+ 4.02
Linoleic	2.44	1.07	- 1.37	2.85	1.23	- 1.62
C <sub>20-22</sub>	0.66	0.53	- 0.13	0.74	0.46	- 0.28
	8.46	21.37	+ 12.91	8.84	15.40	+ 6.56

(b) Pigs at different planes of nutrition to 16 weeks

Pig ... ..	138 (Gilt)			139 (Gilt)		
Planes of nutrition ...	High			Low		
Fatty acids (kg.)	Fed	Deposited	Difference	Fed	Deposited	Difference
Saturated:						
Below C <sub>14</sub>	0.08	Trace	- 0.08	0.02	—	- 0.02
Myristic	0.14	0.13	- 0.01	0.04	0.01	- 0.03
Palmitic	0.87	3.81	+ 2.94	0.32	0.15	- 0.17
Stearic	0.07	1.14	+ 1.07	0.01	0.06	+ 0.05
Unsaturated:						
C <sub>16</sub> (and C <sub>14</sub> )	0.07	0.51	+ 0.44	0.01	0.03	+ 0.02
Oleic	1.97	6.48	+ 4.51	0.75	0.23	- 0.52
Linoleic	0.73	0.38	- 0.35	0.08	0.04	- 0.04
C <sub>20-22</sub>	0.21	0.25	+ 0.04	0.02	0.01	- 0.01
	4.14	12.70	+ 8.56	1.25	0.53	- 0.72

It is an interesting point that the only animal which did not deposit more fat than it ingested is the 16-weeks old pig fed on a restricted ration. At the same time, it will be seen that, even at this very low plane of nutrition, obvious fat synthesis took place during the subsequent stages of growth. The fat deposition in the four 200 lb. weight pigs and in the 16-weeks old pig fed on the high plane of nutrition is not only well marked, but follows the same lines in each case as regards the individual fatty acids which have been laid down.

The amounts of palmitic, oleic and stearic acids in the deposited fats greatly exceed those ingested in the respective diets, and show that the fat synthesized by the animal from non-fatty sources consists predominantly of glycerides of these three acids. The same conclusion applies to the hexadecenoic acid in the depot fats which, though relatively small in amount, is usually several times as much as can possibly have been present in the dietary fat. Myristic acid, however, is present in about the same amount in the animal fats as in the ingested fats, and the evidence as to its origin is therefore inconclusive; unless the absorption of myristic glycerides from the diet was extremely complete, some of this acid must also have been synthesized.

The remaining pig fat component acids—linoleic acid, the unsaturated  $C_{20-22}$  acids and saturated acids below myristic—show an opposite relationship to the respective ingested fatty acids: the amount deposited is consistently less than that present in the diet. It will be noticed that, both in this group and in the preceding group (where evidence of fat synthesis is pronounced), the respective differences for each of the animals show, on the whole, a remarkable and often almost quantitative degree of similarity.

It is clear that fats containing saturated acids of lower molecular weight than myristic acid are neither synthesized nor assimilated by the pig; this accords with the observations of other workers on the body fats of rats and some other animals.

The quantity of linoleic acid in the body fats is not more than (and usually definitely less than) half of that available in the dietary fats; this suggests strongly, although it does not afford positive proof, that, like the rat, the pig is not able to synthesize linoleic acid and derives glycerides of this acid only by assimilation. This view may receive further support from the fact that the octadecadienoic acid of pig depot fats, unlike that of ox depot fat [Hilditch & Longenecker, 1937] has the properties of seed-fat linoleic acid [Hilditch & Stainsby, 1935]. Ellis & Zeller [1930] similarly concluded that, in the pig, linoleic acid is not synthesized, but comes from the food, except possibly in the case of young animals.

The quantity of unsaturated  $C_{20-22}$  acids present as glycerides in the depots likewise falls short of that present in the diet (in this instance in the fish meal constituents); but the disparity is less pronounced than in the case of linoleic acid, and the possibility of some slight degree of synthesis of the acids of this group by the pig (especially in the first weeks of its life) cannot be excluded. For the most part, however, they seem to be derived from the fish meal present in the diet. It was pointed out earlier that the highly unsaturated  $C_{20-22}$  acids in the pig differ in properties from the corresponding acids of the fish meal, and selective absorption of some of the group is perhaps an alternative possibility.

Whilst it cannot, of course, be taken for granted that the increases recorded in Table VII in palmitic, stearic and oleic acids represent the total amounts of each of these acids which have been synthesized, it is of interest to note that the ratios of the increases of palmitic to those of the two  $C_{18}$  acids are as follows:

Pig no.	Palmitic	Stearic + oleic	Palmitic : $C_{18}$ acids
73	6.99	14.37	1 : 2.06
74	5.39	10.04	1 : 1.86
72	4.09	10.21	1 : 2.50
82	2.72	5.93	1 : 2.18
138	2.94	5.58	1 : 1.90

The mean ratio for the whole series is 1:2.08 (wt.) or 1:1.89 (mol.). Palmito- $C_{18}$  glycerides (especially palmitodiolein and palmito-oleostearin) have



previously been shown to be the main components of pig depot fats [Hilditch & Stainsby, 1935], and the present figures point to these as the predominant glycerides in the fat produced by synthesis in this animal.

#### SUMMARY

The component acids of a series of depot (back) fats from pigs fed on different planes of nutrition have been determined. On a restricted diet the deposition of fat is not only slower, but the fat produced is softer, owing to increase in the small proportions of linoleic acid present, together with some increase in the proportion of oleic acid.

Comparison of the amounts of each component acid in the total fats present in the diets with those in the body fats of the animals shows substantial synthesis of glycerides of palmitic, oleic and stearic acids, in the average proportion of 1 mol. palmitic to 1.9 mol.  $C_{18}$  acids. The minor components hexadecenoic and (possibly) myristic acid may also be mainly formed by synthesis, but linoleic and unsaturated  $C_{20-22}$  acids in the depots are derived only from ingested fats.

Our cordial thanks are offered to Dr J. Hammond for his courtesy in providing the experimental material and allowing us to consult his data as regards the diet and the composition of the tissues of the animals, and to Dr E. H. Callow for assistance in the extraction and the determination of the general characteristics of the fats in the feeding-stuffs and in some of the pig depots. The work described in this communication forms part of a research programme undertaken by the Food Investigation Board of the Department of Scientific and Industrial Research, to whom we are indebted for permission to publish the results.

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# LXI. THE DIETARY PREVENTION OF FATTY LIVERS. COMPOUNDS RELATED TO CHOLINE

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THE relative activities of various compounds related to choline in preventing the deposition of fat in the livers of rats maintained on a diet high in fat and low in other lipotropic factors have been studied as a possible source of information as to the mode of action of choline itself [Channon & Smith, 1936; Channon *et al.* 1937; Welch, 1936; Welch & Welch, 1938]. The present paper records similar investigations of a number of other compounds.

Two of the compounds used, cholamine and betaine, had been studied previously by Best & Huntsman [1932]. While cholamine had been found to be inactive, betaine had prevented the deposition of liver fat, a result confirmed by Welch & Welch [1938]. The experiments of Best & Huntsman, however, were carried out with the 40 % fat-60 % grain diet and it seemed desirable to repeat the work using a "synthetic" choline-free diet; moreover, the latter authors provided no evidence regarding the relative activity of betaine in terms of choline. Welch & Welch [1938] have since suggested that it has rather more than half the activity of choline. In view of the fact that liver tissues can oxidize choline to betaine aldehyde and probably to betaine [Mann & Quastel, 1937; Bernheim & Bernheim, 1938; Mann *et al.* 1938], the degree of activity of betaine is a point of some importance since it might possibly be the compound ultimately active when choline supplements prevent liver fat deposition.

Another compound tested was creatine, since several authors have suggested that choline is a precursor of creatine in metabolism [Riesser, 1913; 1914; Baumann & Hines, 1918; Shanks, 1921; Klee & Petropoliades, 1928]. Finally, two further compounds related to choline were investigated: choline methyl ether and tetra( $\beta$ -hydroxyethyl)ammonium chloride. Since choline might act by enabling lecithin formation to take place it was decided to study the methyl ether of choline, to see whether etherification of the alcohol group robbed the compound of its lipotropic activity. The results with the tetrahydroxy derivative should show whether the alkyl groups were necessary for the activity found in choline and some of its analogues.

## EXPERIMENTAL

*Choline methyl ether* was prepared by a method based on that described by Renshaw [1910] for choline.  $\beta$ -Chloroethyl methyl ether, prepared from the monomethyl ether of ethylene glycol by the action of phosphorus pentachloride, was sealed with a molecular proportion of trimethylamine in a bomb tube and heated at 95° for 6 hr. with occasional shaking. The contents of the tube were then dissolved in alcohol, purified by treatment with charcoal and precipitated by means of acetone and ether. The crystallized material was purified by similar means. Exact analysis was rendered difficult by its extremely hygroscopic

nature but the chloroaurate was a stable compound and had m.p. 155°. (Found: C, 15.90; H, 3.59; Au, 43.38%.  $C_6H_{18}ONAuCl_4$  requires C, 15.75; H, 3.51; Au, 43.15%.)

The tetra( $\beta$ -hydroxyethyl)ammonium chloride used in these experiments was a sample kindly given to us by Mr A. L. Bacharach.

#### *Animal feeding and liver analyses*

Groups of 10 rats were fed on a basal diet producing a fatty liver, one group serving as a control and the others receiving supplements of the various compounds or of choline itself, the supplements in aqueous solution being mixed in the diet. The choline content of the basal ration was negligible except in exp. 5 when marmite was included to supply vitamin B complex, and provided about 1.5 mg. choline per rat per day, the effect of which, it is considered, would be negligible. Except in exp. 5, the individual livers were analysed by digesting with 40% NaOH and refluxing with alcohol, the fatty acids and unsaponifiable fraction then being extracted with ether and subsequently purified by treatment with light petroleum: in these cases "fat" represents fatty acids plus unsaponifiable matter. In exp. 5 the pooled livers were ground with sand and extracted with alcohol and ether and in this case therefore "fat" represents the total ether-soluble material so obtained. Details of the diets differed in the various experiments and are considered when discussing the results.

#### RESULTS

*Betaine.* The basal diet used in these experiments consisted of beef dripping 40, protein 8, glucose 46, salt mixture 5, cod-liver oil 1 part. In addition aneurin was added to the diet so that each animal received about 10  $\mu$ g. daily. The protein fraction was egg albumin in exps. 1 and 3 and caseinogen (alcohol-ether-extracted) in exp. 2. In exps. 1 and 3 the animals were all bucks while in exp. 2 there were 5 bucks and 5 does in each group. Betaine hydrochloride and choline chloride were used but the amounts are expressed in terms of the free bases.

The first two experiments were, of necessity, preliminary in nature, since none of the figures recorded in the literature was of any assistance in forming an estimate as to the lipotropic activity of betaine relative to that of choline. All the relevant data are presented in Table I together with those of exp. 3.

On the animal side, both exps. 1 and 2 are obviously satisfactory, the weight losses not being such as to interfere with the interpretation of the results.

In the first experiment 0.1% choline and betaine were given to two groups respectively and it can be seen that while the former supplement has prevented a large increase in the amount of fat deposited in the liver, the presence of 0.1% betaine has not had a very marked effect. The livers of the control animals contained 27.01% fat, and those of the choline group 8.18%, values to be contrasted with the 22.14% present in the livers of the animals receiving betaine. In our experience, the difference between the control and betaine groups is hardly significant. The figures for the amount of fat in the liver of the 100 g. rat, 1.82 and 1.28 g., show, however, a larger difference and it was accordingly decided to increase the betaine and to reduce the choline dosage in the next experiment.

Before considering the results obtained in exp. 2, it must be pointed out that the wide difference between the two control values for liver fat obtained in exps. 1 and 2 is due to the fact that in the first, egg albumin was the basal protein, while in the second it was caseinogen; this point has already been discussed by Channon *et al.* [1938]. From the results of exp. 2, it can be seen that

Table I. *Effect of betaine supplements in preventing liver fat deposition*

Group	No. of rats	Change in wt. $\pm$ % initial body wt.	Liver % final body wt.	Fat % liver wt.	Fat in liver of 100 g. rat g.	Base intake mg./rat/day	Choline equivalent mg./rat/day
Exp. 1. Period of feeding 18 days:							
1. Control	8	+ 4.8	6.6	27.01	1.82	Not recorded	
2. +0.1% choline	10	+ 5.0	5.2	8.18	0.41		
3. +0.1% betaine	10	+ 8.6	5.4	22.14	1.28		
Exp. 2. Period of feeding 17 days:							
4. Control	9	- 9.1	3.9	17.03	0.70	Not recorded	
5. +0.03% choline	10	- 6.3	3.9	15.51	0.65		
6. +0.05% choline	10	- 5.1	3.6	9.58	0.36		
7. +0.20% betaine	10	- 7.9	3.3	8.50	0.28		
8. +0.50% betaine	10	- 5.1	3.5	4.80	0.17		
Exp. 3. Period of feeding 18 days:							
9. Control	10	+ 1.3	4.8	24.10	1.24	---	---
10. +0.03% choline	10	0	4.1	15.70	0.71	2.8	2.8
11. +0.05% choline	10	+ 1.0	3.8	13.18	0.51	4.4	4.4
12. +0.07% choline	10	- 2.2	3.7	8.79	0.32	6.5	6.5
13. +0.10% betaine	10	+ 1.8	4.1	16.49	0.76	9.4	8.4
14. +0.15% betaine	10	+ 2.2	4.1	15.23	0.68	14.1	12.7
15. +0.20% betaine	10	- 1.0	3.7	12.41	0.48	17.2	15.4

the groups 6, receiving 0.05 % choline, and 7, receiving 0.20 % betaine, have given practically the same values for liver fat, 9.58 and 8.50 %, the control figure being 17.03 %; the absolute amounts support this conclusion. It is to be noted that in this experiment the inclusion of 0.50 % betaine has maintained the liver fat at nearly the normal level, 4.80 % and 0.17 g. in the 100 g. rat. This may be of significance in view of the fact that on several occasions even massive doses of choline have failed to show so marked an action in preventing fatty infiltration.

These results suggested that betaine had a little more activity than 25 % of its weight of choline in preventing fat deposition in the liver, a conclusion which may be better expressed by saying that it is slightly more active than 32 % of an equimolar amount of choline. Exp. 3 was designed to test this finding and seven groups of animals were used, six of them receiving graded quantities of choline and betaine as set out in Table I. The food intakes of the various groups were measured in order to show exactly the intake of base in each group.

In exp. 3 the weight changes and food intakes (8.6–9.4 g./rat/day) are comparable in all the groups. The figures for both the percentage of fat in the liver and the weight in the liver of the 100 g. rat show that 4.4 mg. choline and 17.2 mg. betaine have had substantially the same preventive action, the results being 13.18 % and 0.51 g., 12.41 % and 0.48 g. respectively, as compared with the control values of 24.10 % and 1.24 g. In Table I the amounts of betaine administered are converted into their equivalents in terms of choline, and comparing the choline and betaine intakes in this form the results of groups 11 and 15 indicate a 29 % activity for betaine. There is no significant difference between the groups which received 9.4 mg. and 14.1 mg. betaine, a fact which can only be referred to the individual variations among the animals. However, the values obtained for liver fat, 16.49 and 15.23 % respectively, are similar to that obtained in the case of 2.8 mg. choline, 15.70 %, while 100 g. rat figures for the three groups are 0.76, 0.68 and 0.71 g. respectively. The activity of betaine in these cases is 33 and 22 % of that of choline. From the approximate value of 32 % suggested by the results of exp. 2, and the values 29, 33 and 22 % obtained

in this more accurate experiment, it is reasonable to conclude that betaine has about 30 % of the efficiency of choline in preventing fatty infiltration into the liver under these conditions.

*Cholamine and creatine.* The groups of animals used in this experiment consisted entirely of bucks and the same control group served for this experiment as for exp. 1. Two groups received, in addition to the basal 8 % egg albumin diet, 1 % cholamine and 0.5 % creatine respectively.

Table II. *The effect of cholamine and creatine in fatty liver prevention*

Group	No. of rats	Change in wt. $\pm$ % initial body wt.	Liver % final body wt.	Fat % liver wt.	Fat in liver of 100 g. rat g.
Exp. 4. Period of feeding 18 days:					
1. Control	8	+ 4.8	6.6	27.01	1.82
16. + 1 % cholamine	9	+ 4.1	6.0	25.64	1.67
17. + 0.5 % creatine	10	+ 1.1	6.4	27.96	1.71

Clearly neither compound has exerted any lipotropic action, for the groups of animals receiving them have livers as fatty as those of the control group, and in view of the relatively large quantities administered, it was not considered profitable to pursue the investigations any further.

*Choline methyl ether and tetra( $\beta$ -hydroxyethyl)ammonium chloride.* In view of the considerable toxicity which the choline analogues previously investigated had been found to possess [Channon & Smith, 1936; Channon, Platt & Smith, 1937], it was decided to use relatively small quantities of these two compounds in preliminary experiments. The tetrahydroxyethyl derivative was tested first and two groups of does received a basal diet calculated to produce the "cholesterol" fatty liver: caseinogen (alcohol-ether-extracted) 5, beef dripping 30, glucose 52, marmite 5, salt mixture 5, cholesterol 2, cod liver oil 1 part. One group served as control and the other received a supplement of 0.14 % of the tetrahydroxyethyl derivative. The experimental data for this and the subsequent exps. 6, 7 and 8 are presented in Table III.

Table III. *Effect of choline methyl ether and tetra( $\beta$ -hydroxyethyl)ammonium chloride in fatty liver prevention*

Group	No. of rats	Change in wt. $\pm$ % initial body wt.	Liver % final wt.	Fat % liver wt.	Fat in liver of 100 g. rat g.
Exp. 5. Period of feeding 14 days:					
18. Control	10	- 2.3	5.5	24.3	1.34
19. + 0.14 % tetrahydroxyethyl derivative	9	+ 1.0	5.2	29.4	1.52
Exp. 6. Period of feeding 12 days:					
20. Control	10	- 11.3	4.1	16.36	0.68
21. + 0.10 % choline	10	- 9.6	3.5	7.74	0.30
22. + 0.11 % choline methyl ether	9	- 13.6	4.1	17.00	0.74
23. + 0.25 % tetrahydroxyethyl derivative	10	- 10.7	4.3	15.76	0.67
Exp. 7. Period of feeding 18 days:					
1. Control	8	+ 4.8	6.6	27.01	1.82
2. + 0.10 % choline	10	+ 5.0	5.2	8.18	0.41
24. + 0.28 % choline methyl ether	10	+ 1.7	5.6	24.46	1.41
Exp. 8. Period of feeding 17 days:					
4. Control	9	- 9.1	3.9	17.03	0.70
6. + 0.05 % choline	10	- 5.1	3.6	9.58	0.36
25. + 0.50 % choline methyl ether	9	- 14.3	3.6	16.32	0.62

The figures for weight change show that the compound is in no way toxic, while from the analytical figures it appears to have had little action on liver fat deposition. Actually the percentage of fat in the livers of the group receiving the tetrahydroxyethyl derivative, 29.4, is higher than in the control group, 24.3, but the difference in the absolute weights, which are 1.52 and 1.34 g., is not so great. While these groups do not differ significantly, further evidence as to the effect of a larger dose of the tetrahydroxyethyl derivative was clearly desirable, and accordingly this compound was again fed in exp. 6 in which choline methyl ether was also studied.

Four groups of does were maintained on a basal diet consisting of caseinogen (alcohol-ether-extracted) 5 parts, beef dripping 40, glucose 49, salts 5, cod liver oil 1 part, each animal receiving in addition 10  $\mu$ g. aneurin daily. Two groups received 0.11 % choline methyl ether and 0.25 % tetra( $\beta$ -hydroxyethyl)-ammonium chloride respectively, while a third group received 0.10 % choline to facilitate the assessment of lipotropic action.

The weight losses in exp. 6, although rather large, are not such as to interfere with any conclusions which may be drawn, and from the point of view of weight maintenance, the superiority of egg albumin as a basal protein in these low-protein diets is clearly demonstrated (compare groups 1 and 9 with 4 and 20). From study of these results it is seen that the tetrahydroxyethyl derivative has no choline-like action on liver fat, nor has the methyl ether in the amount employed. The percentages of liver fat in these two groups, 15.76 and 17.09 respectively, are substantially the same as the control group's 16.36 %, a conclusion borne out by the values obtained for the weights in the 100 g. rat, 0.68, 0.74 and 0.67 g., for the control, choline methyl ether and tetrahydroxyethyl compound groups respectively. In contrast with these, group 21, which received 0.10 % choline, had only 7.74 % liver fat or 0.30 g. in the 100 g. rat, figures which demonstrate very clearly the powerful action of choline in preventing the deposition of liver fat under these conditions.

Clearly tetra( $\beta$ -hydroxyethyl)ammonium chloride had no lipotropic action but the lack of toxicity of the choline methyl ether made it desirable to study its action in large dosages, and two further experiments were therefore carried out using this derivative with the results quoted in Table III. In exp. 7 the animals used were bucks and the basal diet consisted of egg albumin 8, beef dripping 40, glucose 46, salt mixture 5, cod liver oil 1 part and 10  $\mu$ g. aneurin per rat per day. The same diet was used in exp. 8 with the substitution of caseinogen for egg albumin as the protein while in this case the animals consisted of equal numbers of both sexes.

Considering the results of exp. 7 first, it would appear just possible that the choline ether possesses some activity, for although the results obtained for this group, 24.46 % and 1.41 g., are not markedly different from the control values, 27.01 % and 1.82 g., nevertheless they are somewhat lower. In view of the marked effect of 0.1 % choline, which has prevented an increase in fat content of nearly 19 % of the wet liver weight, any action of the ether must be slight and it was therefore decided to use a massive dosage, 0.5 %, in the next experiment.

In exp. 8, the choline methyl ether has clearly exercised no lipotropic action whatsoever, the values both for the percentage of liver fat and for the weight in the 100 g. rat not differing significantly from those of the control group. On the other hand, only 0.05 % choline has had a marked preventive action. The negative result obtained with this large dose provides confirmation of the conclusion from exps. 6 and 7 that choline methyl ether has no activity in preventing this fatty infiltration into the liver.

## DISCUSSION

In the present work betaine has been found to possess about 30% of the activity of choline in preventing fatty infiltration in the livers of rats maintained on choline-free diets of high fat and low protein contents. This finding that betaine is lipotropically active confirms previous studies by Best & Huntsman [1932] and by Welch & Welch [1938], the latter authors also finding betaine aldehyde to be active. Neither paper, however, offers evidence as to the relative efficiencies of the two compounds although Welch & Welch [1938] suggest that betaine may have more than 50% of the activity of choline, but defer a definite statement until a later date. Quastel and his associates [1937; 1938] and Bernheim & Bernheim [1938] having shown that choline is oxidized to betaine aldehyde and then probably to betaine by surviving rat liver tissue and by enzyme systems extracted from rat liver, the present finding that betaine is less active than choline in fatty liver prevention suggests that, among other possibilities, it may be first converted into choline in the liver, this choline then being available for use: if this should be so, it would provide an interesting example of a readily reversible reaction taking place in animal tissues. Another explanation may be that while betaine itself is the active material, its activity is fully manifested only when it is produced in the liver by the oxidation of choline *in situ*, a part only of any dietary betaine being available and the rest oxidized before it is able to exercise its lipotropic action.

The very definitely negative result obtained with cholamine in exp. 4 confirms the work of Best & Huntsman [1932] and shows that if, in fatty liver prevention, lecithin formation resulting from choline feeding is the means whereby choline acts, the analogous synthesis of kephalin under similar conditions either plays no part, or cannot be influenced by cholamine feeding. This conclusion may indicate that kephalins have little or no importance in the metabolism of dietary fatty acids.

The results of the experiment with creatine indicate that this compound cannot serve as a source of choline in the body.

The results obtained with choline methyl ether show that if the primary alcohol group is etherified, choline becomes inactive. Apparently the body cannot remove a methyl ether group of this type although it appears to be able to demethylate methionine quite readily. Here again no light is thrown on the way in which choline may act, because, while the inactivation may be due to the fact that the choline ether can no longer be linked up in a phosphatide molecule, etherification may easily have inactivated the compound, whatever its mode of action.

Finally, study of tetra( $\beta$ -hydroxyethyl)ammonium chloride has revealed the necessity that the alkyl radicles of the choline molecule be intact for the possession of lipotropic properties, a finding in agreement with the complete lack of activity of cholamine.

## SUMMARY

1. Studies have been made of five compounds—betaine, cholamine, creatine, choline methyl ether and tetra( $\beta$ -hydroxyethyl)ammonium chloride—in order to discover whether they possess choline-like properties in preventing the deposition of liver fat under certain dietary conditions.

2. Of these compounds, only betaine was found to possess lipotropic properties. Its activity in fatty liver prevention was estimated to be approximately 30% of that of choline.

3. The relationship of these results to the theory that choline acts by enabling lecithin formation to take place is discussed.

I wish to record my indebtedness to Prof. H. J. Channon for helpful criticism and advice; and to Mr A. L. Bacharach for the gift of the tetra( $\beta$ -hydroxyethyl)-ammonium chloride. The expenses of this research were defrayed in part by a grant to Prof. H. J. Channon from the Medical Research Council, to whom I wish to make grateful acknowledgement.

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## LXII. THE INFLUENCE OF PHYTIN AND OF FATS ON THE PRODUCTION OF RICKETS BY A CEREAL DIET

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IN an earlier paper [1937] we showed that the tendency of cereals to produce rickets entirely disappeared when calcium lactate was added so as to make the Ca/P ratio 1·0 : 0·5.

Other explanations for the rachitogenic action of cereals have been put forward. Bruce & Callow [1934] considered that the phosphorus (approximately one-half) which is combined as phytin in cereals is not available. McCance & Widdowson [1935] estimated the amount of phytin in 64 foodstuffs and by means of feeding experiments on four human subjects, using phytin obtained from Messrs Ciba Ltd., estimated that 20–60% of the phytin ingested was excreted unchanged in the faeces.

In the present series of experiments the authors have used similar phytin from Messrs Ciba Ltd., adding it in varying proportions to a diet consisting otherwise entirely of cereals; these diets have then been fed to white Wistar rats. At the same time for comparison other rats were fed on the same diet without added phytin but with varying amounts of calcium lactate added to it.

The experiments consisted of feeding these diets to the rats for 5 weeks, noting the increase in weight and then, after killing the rats, estimating the amount of rickets by examination of the bones stained with silver nitrate. Projection drawings of the stained bones were made and four categories were recognized, (1) severe rickets (+++), (2) rickets(++), (3) slight rickets(+), and (4) normal bones (0).

Additional information was obtained by determining the amount of bone ash in the dry, fat-extracted bones. By this means it was thought that any tendency of the phytin-P to produce rickets or prevent the proper formation of bones would be disclosed.

A summary of the results of these experiments is given in Table I.

A consideration of these results shows at once that the added phytin has no prohibitive action on bone formation. On the contrary the addition of 1·5% greatly reduces the rickets and 5% completely prevents it. With a gradual increase of the amount added, and consequent reduction of the disproportion of P to Ca in the diet, we have a gradual improvement in the character of the bone and a rise in the ash content.

Comparison, with the results obtained by the addition of calcium lactate is best made by first considering the Ca/P ratios and then the percentage of each substance added. Thus, for equal additions of 0·75% of calcium lactate and phytin (nos. 2 and 3), the ratio in one case is 1 : 2·5 and in the other 1 : 3·5 and we have slight and severe rickets and bone ash percentages of 40·5 and 34·6. If, however, we consider the ratio 1 : 2·5 we find that with the addition of 0·75%

Table I

Diet	Av. initial wt. of 3 rats g.	Av. gain of 3 rats g.	Bone condi- tion at end of test	Bone ash %	Ca : P ratio calculated	Ref. no.
*Germ flour 100 %	50	17	+ + + + + + + + +	28.3	1 : 9	1
+ 0.75 % Ca lactate	48	57	+ + +	40.5	1 : 2.5	2
+ 0.75 % phytin	48	17	+ + + + + + + +	34.6	1 : 3.5	3
+ 1.5 % Ca lactate	48	64	0 0 0	50.68	1 : 1.4	4
+ 1.5 % phytin	48	45	+ 0 0	40.4	1 : 3.0	5
+ 3.0 % Ca lactate	55	90	0 0 0	60.7	1 : 0.45	6
+ 3.0 % phytin	48	51	+ 0 0	43.0	1 : 2.5	7
+ 5.0 % phytin	58	55	0 0 0	52.8	1 : 2.0	8
+ 3.0 % Ca lactate + 5 % phytin	55	71	0 0 0	56.3	1 : 1.3	9

\* The germ flour used consisted of a mixture of 75 % white flour and 25 % sterilized wheat germ.

calcium lactate we get slight rickets and bone ash of 40.5 % and by the addition of 3 % phytin to produce a similar ratio we get slight rickets and bone ash of 43.0 % (No. 7). These amounts of calcium lactate and phytin represent in the case of lactate an addition of 0.1 g. of Ca and in the case of phytin an addition of 0.36 g. Ca. This actually tends to confirm our previous contention that it was the disproportion of P to Ca rather than the presence of any other substance which caused the rickets, especially if one considers experiment No. 9 where an addition of 3 % calcium lactate and also 5 % phytin was made. Here the additions of lactate and phytin made are equivalent to 1.0 g. Ca and 1.05 g. P to a diet containing 0.04 g. Ca and 0.36 g. P, resulting in a Ca/P ratio of 1 : 1.3. The results obtained were good but not equal to those of experiment No. 6 where only lactate was added, equivalent to an addition of 0.4 g. Ca and no P and resulting in a Ca/P ratio of 1 : 0.43, which is much closer to the optimum ratio of 1 : 0.5.

If, as is suggested by McCance & Widdowson [1935], 20–60 % of the P in phytin is unavailable, the Ca/P ratios for experiments Nos. 7 and 9 might be assumed to be 1 : 1.5 and 1 : 0.7. The results, however, indicate that a much higher percentage of the phytin-P is utilized.

A recent paper by McDougall [1938] suggests that the additions of fats, olive oil or lard, prevents the occurrence of rickets on a cereal diet by creating favourable conditions for the absorption of Ca. It is inferred that an insoluble calcium phosphate is precipitated in the intestine by the action on phytin of an enzyme

which splits it into soluble sodium phosphate which then reacts with Ca to form insoluble calcium phosphate.

The above results did not confirm this suggestion and further experiments were started to see whether it would be possible to obtain results similar to McDougall's by the addition of fat to the diet. In these experiments mixtures of germ flour, casein and varying proportions of calcium lactate were made. These were fed to rats with the addition in the experiments indicated of 10% of vitamin-free hardened vegetable oil. Vitamin-free oil was chosen in order to remove any possibility of the results being affected solely by vitamin D and not by the fatty acids of the oil.

After 5 weeks the rats were killed and the bones examined for rickets as before. Table II shows the results obtained.

Table II

Diet (%)	Initial wt. of rat g.	Gain in wt. g.	Bone condition at end of test	Bone ash %	Ca : P ratio calculated	Ref. no.
Germ flour 73.5	50	100	0	Not available	1 : 1.2	1
Casein 24.5	50	95	0			
Ca lactate 2.0	45	105	0			
Germ flour 66.0	50	90	0	Not available	1 : 1.5	2
Casein 22.0	50	90	0			
Ca lactate 2.0	55	80	0			
Hard. veg. oil 10.0						
Germ flour 73.7	50	90	0	54.6	1 : 1.3	3
Casein 24.5	50	60	0			
Ca lactate 1.75	55	80	+			
Germ flour 66.15	50	90	++	53.1	1 : 1.4	4
Casein 22.10	50	60	+			
Ca lactate 1.75	Found dead					
Hard. veg. oil 10.0						
Germ flour 74.25	50	90	++	46.5	1 : 1.4	5
Casein 24.25	50	50	0			
Ca lactate 1.5	55	125	+++			
Germ flour 66.5	55	80	++	47.5	1 : 1.5	6
Casein 22.0	45	50	++			
Ca lactate 1.5	45	55	+			
Hard. veg. oil 10.0						
Germ flour 74.5	60	70	+	52.3	1 : 1.8	7
Casein 24.5	55	65	+			
Ca lactate 1.0	60	50	+			
Germ flour 74.5	50	55	+++	47.2	1 : 2.5	8
Casein 25.0	50	60	+++			
Ca lactate 0.5	50	45	++			
Germ flour 67.0	50	50	++	47.5	1 : 2.5	9
Casein 22.5	50	50	+++			
Ca lactate 0.5	50	60	+			
Hard. veg. oil 10.0						
Germ flour 75.0	45	50	+	37.3	1 : 3.2	10
Casein 25.0	45	60	+			
	50	40	+			
Germ flour 67.5	50	40	+++	29.1	1 : 3.5	11
Casein 22.5	45	40	+++			
Hard. veg. oil 10.0	50	45	+			

These results show no decided prevention of rickets by the addition of oil, yet such experiments in which a diet is given to produce not severe rickets but mild or moderate rickets, ought easily to show the preventive action of oil. The

experiments show that, when such diets are given, to obtain consistently normal bones not less than 2% of calcium lactate must be added representing a calculated Ca/P ratio of 1 : 1.2.

#### SUMMARY

1. Our previous finding is confirmed that the tendency of cereals to produce rickets resides in their low Ca and high P contents, and that this tendency can be entirely counteracted by adding calcium lactate so as to make the Ca/P ratio 1 : 0.5.

2. The rachitogenic action of cereals does not reside in the phytin which they contain, for the reason that the addition of phytin instead of increasing this action reduces the rickets.

3. The addition of a vegetable oil free from vitamin D does not prevent the occurrence of rickets resulting from a cereal diet; here we fail to confirm the findings of McDougall.

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# LXIII. SOME PROPERTIES OF THE REDUCING MATERIAL IN CERTAIN FRACTIONS OF NORMAL URINES

## III. SOME OBSERVATIONS ON THE NATURE OF THE NON-FERMENTABLE REDUCING SUBSTANCES IN "FASTING" URINE

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IN the two preceding papers of this series [Harding *et al.* 1936, 1, 2] the nature of the "free fermentable sugar" in urine and of the substances which yield fermentable sugar on hydrolysis was discussed. No attempt was made at that time to determine the nature of the reducing substances which were non-fermentable even after hydrolysis. Everett & Sheppard [1930; 1931] and Everett *et al.* [1934, 1] presented evidence indicating the presence of a substance which they named uroketose. It is precipitated by neither  $\text{Hg}^{++}$  nor Ba salts in neutral solutions. It is resistant to bromination at room temperature and gives rise to 2-furfuraldehyde when heated with acid. Everett claims that this substance, which differs from ketoxylose, constitutes the greater part of the reducing substance of normal urine.

It was thought that further information as to the nature of the residual reducing substances in normal human urine might be gained by the application of the modification of Everett *et al.* [1934, 1] of the Votocek and Nemecek technique of Br oxidation and a modified NaOI oxidation method to urine fractions obtained by the copper-lime method [Archibald, 1935; Harding *et al.* 1936, 1] which gives solutions of the urine sugars free from nitrogen and phenols. These procedures were therefore first tested on dilute sugar solutions of about the same concentration as the reducing substances found in the urine fractions and then applied to the urine fractions.

### *Analytical methods*

Composite fasting urines were collected, diluted and treated with Pb acetate [Harding *et al.* 1936, 1, 2],  $\text{HgSO}_4$  [West *et al.* 1929] and copper-lime [Harding *et al.* 1936, 1, 2].<sup>2</sup> Further fractionation was accomplished by acid hydrolysis and NaOI oxidation according to the steps illustrated in the diagram.

<sup>1</sup> Part of the data embodied in this paper was taken from a thesis submitted by one of the writers (R. M. A.) in partial fulfilment of the requirements for the degree of Ph.D. in the University of Toronto.

<sup>2</sup> In view of the fact that urine may contain considerable amounts of diastase, starch-free filter papers were used throughout these experiments as well as in those previously reported.

Six 4 hr. fasting normal urines—Dilution 1·14		
Basic Pb acetate—Dilution 5		
HgSO <sub>4</sub> -BaCO <sub>3</sub> —Dilution 10		
Evaporate under reduced pressure		
Fraction A—Dilution 3		
Cu-lime (at dilution 0·380)		
Cu-lime precipitate		Cu-lime filtrate
Fraction B—Dilution 2·5		Fraction G—Dilution 1·0
Hydrolysis—Fraction C—Dilution 2·74	Hydrolysis	Hypoiodite
	Cu-lime	Hydrolysis
Hypoiodite—Fraction F		Cu-lime
Dilution 3·42		
Hypoiodite—Fraction D—Dilution 3·17	Precipitate	Precipitate
Hydrolysis—Fraction E	Fraction H	Fraction J
Dilution 3·47	Dilution 0·346	Dilution 1·38

Dilutions reported are based on the assumption that 100 ml. of urine per 4 hr. corresponds to Dilution 1.

Hydrolyses were carried out by boiling in  $N$  H<sub>2</sub>SO<sub>4</sub> under a reflux condenser for 1 hr. NaOI oxidations were done by the method described by Harding *et al.* [1936, 2] except that the final dilutions were varied and the small amount of KI left after HgSO<sub>4</sub> treatment was precipitated as AgI by the addition of a slight excess of powdered Ag<sub>2</sub>SO<sub>4</sub>, the excess Ag being removed by the addition of a slight excess of HCl. The copper-lime filtrates required increased amounts of I<sub>2</sub> solution and of NaOH.

All fractions were adjusted to pH 6·5. "Reducing values" were determined by the Harding & Downs [1933] reagent and are expressed as mg. glucose per single 4 hr. specimen. Br oxidation curves were determined on Fractions B, C, D, E and F by the method of Everett *et al.* [1934, 1], using the Harding & Downs reagent to determine changes in reducing value. The quantity of furfural-yielding material was determined in Fractions B, C, D and E by McCance's [1926] technique, separating the two layers by centrifuging for 30 sec. instead of by standing, and the free sugar by differential fermentation [Harding & Nicholson, 1933] in Fractions B, C, E, H and J.

#### Discussion of methods

Although most of the solutions used were obtained by regenerating the precipitates after a single treatment with copper-lime and therefore contained small amounts of nitrogenous material [Archibald, 1935], determinations done on N-free solutions obtained by reprecipitating the material [Archibald, 1935; Harding *et al.* 1936, 1] gave parallel results. It is therefore certain that none of the phenomena observed can be due to N-containing substances.

NaOI rapidly destroys all the "reducing power" of common aldohexoses and aldopentoses and the furfural-producing properties of the latter; it is therefore a more satisfactory method than Br oxidation for differentiating ketose from aldoses such as mannose, which are oxidized but slowly by the Br. Everett *et al.* [1934, 1] report that using Kolthoff's method of NaOI oxidation in urines, 30 % of the uroketose and added fructose is destroyed. It has been found, however, that the reducing power of fructose towards the Harding-Downs reagent, both in water solution and when added to urine, is almost unchanged by the NaOI or Br procedures used here. NaOI oxidation also completely removes the "reducing values" of free glucuronic acid, glucuronic acid monobenzoate, and borneol glucuronate both before and after hydrolysis as well as after 48 hr. treatment with Br.

The estimation of the amounts of furfural-producing substances present was of necessity very inaccurate because of the great differences in furfural yield between even such closely related substances as pentoses, e.g. arabinose yields three times as much furfural as does xylose.

In 48 hr. Br neither hydrolyses nor oxidizes appreciable quantities of glucuronic acid monobenzoate but it does hydrolyse 40% of borneol glucuronate and destroys the reducing properties of the material thus liberated. Repeated Br oxidations on sugars 100 times as dilute as those used by Everett *et al.* [1934, 1] gave curves identical with those obtained by these authors. The Br oxidations gave results which fitted very well into a smooth curve. Little difficulty was experienced in repeating the results with a given fraction if the conditions influencing the rate of oxidation were carefully controlled.

*The nature of the non-fermentable reducing substances of "fasting" urine*

Typical results for the changes in reducing value towards the Harding-Downs reagent induced by NaOI oxidation and for the fermentable sugar in the various urine fractions are given in Table I. Fig. 1 represents the Br oxidation

Table I. *The fermentable sugars and the reducing substance destroyed by NaOI in various urine fractions*

	B	C	Increase	E	Increase	H	J
Total reduction	26.8	32.3	5.5	9.7	2.8	6.1	4.3
Fermentable sugar (including galactose)	10.3	16.6	6.3	3.0	3.0	1.8	1.0
Reducing material destroyed by NaOI	19.9	28.8	8.9	—	—	—	—

All results are expressed as mg. glucose/4 hr.

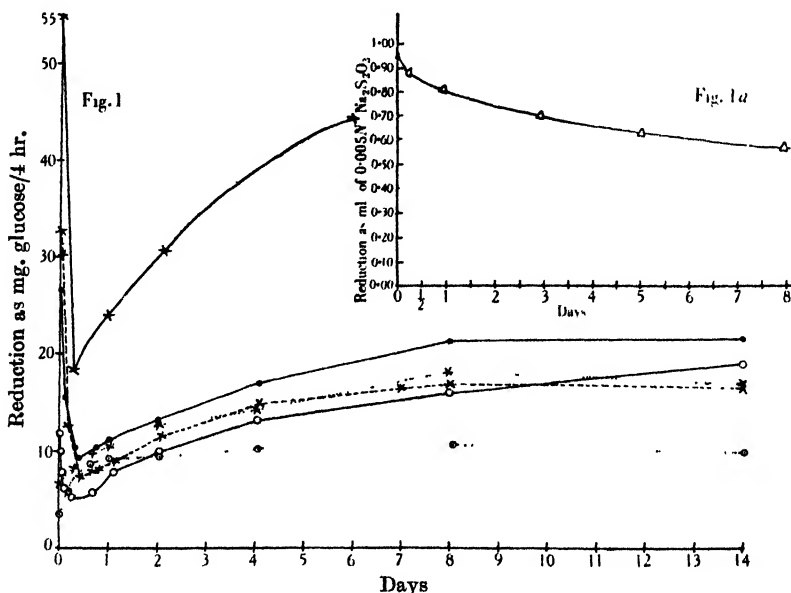


Fig. 1. The effect of Br oxidation on the reducing power of different urine fractions. Fraction B,  $\bullet-\bullet$ ; Fraction C,  $\times-\times$ ; Fraction D,  $\times\cdots\times$ ; Fraction E,  $\circ-\circ$ ; Fraction F,  $\circ\cdots\circ$ ; HgSO<sub>4</sub>-KOH filtrate,  $\times-\times$ .

Fig. 1a. The effect of Br oxidation on the reducing power of glucuronic acid monobenzoate.

curves plotted over a period of 14 days and the curves obtained during the first 16 hr. of oxidation are recorded on a larger scale in Fig. 2. Although these are results obtained with male urines, composite specimens of female urine gave similar results.

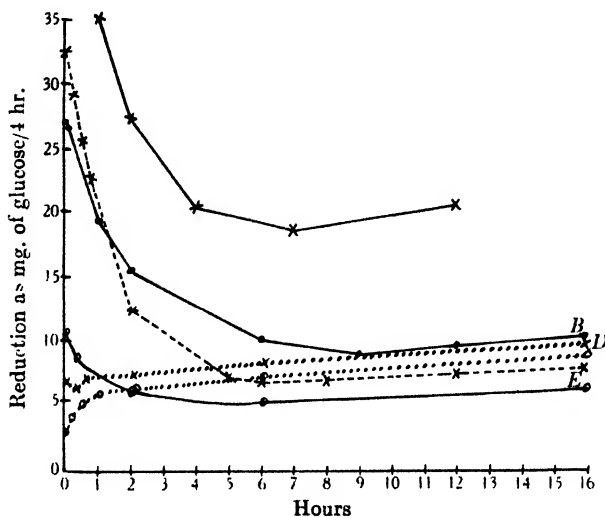


Fig. 2. For description see Fig. 1.

NaOI oxidation decreases the reducing values of Fraction B from 26.8 mg./4 hr. to 6.9 mg./4 hr. and of Fraction C from 32.3 mg./4 hr. to 3.5 mg./4 hr. Of the reducing material destroyed by NaOI in Fraction B, 50% can be accounted for by the fermentable sugar present and of that destroyed in Fraction C, 57%. This is a much greater proportionate drop in the reducing power than that observed by Everett *et al.* [1934, 1], but the difference between the two observations is to be expected when the much more drastic clearing procedure used here is considered, the copper-lime precipitation giving a fraction which is much more nearly a solution of carbohydrate only. Furfural-yielding materials represent a considerable portion of the aldose-like substance, the percentage depending on their reducing power. Expressed in terms of xylose the total furfural-producing material in B represents about 7 mg./4 hr. of which about two-thirds are destroyed by NaOI. The nature of the furfural-producing material has not been definitely determined. Although some of it may be glucuronic acid which French workers [see Cammidge, 1913; 1923] have long maintained is responsible for the non-fermentable reducing material of urine, a considerable part of it at least is probably pentose, since three yeasts, *M. tropicalis*, *M. pinoyi* and *M. metalondinensis*, which were found to have the ability to remove small amounts of pentose (xylose, arabinose, ribose and rhamnose), removed reducing material from urine fractions which had been freed from fermentable hexoses by *S. marxianus*, which does not attack pentose. Fraction G (the copper-lime filtrate) could not be analysed by mycological methods as it contained small amounts of material which were removed erratically by all the yeasts including baker's yeast.<sup>1</sup> However, yeast treatment of Fractions H and J shows "pentose"-like material which is equal in amount in the two fractions. That NaOI treat-

<sup>1</sup> This finding supports the conclusion of Harding & Selby [1933] that normal urine contains small amounts of reducing substances other than glucose which are removable by yeast.



ment did not destroy this material in Fraction G may be due to ketonic properties or to protection from oxidation by combination with some hydrolysable radical. Van Ekelen *et al.* [1933], Birch [1934] and many later workers have presented evidence showing the presence of ascorbic acid in urine, but the alkalinity of the clearing reagents and the low recovery of reducing material after copper-lime precipitation of ascorbic acid preclude the possibility that any appreciable fraction of the furfural-producing material in our fractions can be derived from vitamin C. That hydrolysis of Fraction G liberates reducing materials which on separation by copper-lime precipitation show properties similar to those in Fraction B suggests that the precursors are unfamiliar substances since the higher polysaccharides are removed by the preliminary clearing and mono- and di-saccharides are precipitated by copper-lime. The resistance of part of the furfural-producing material to NaOI is not due to protection from oxidation by a hydrolysable radical as Fraction F gives rise to fully as much furfural as does Fraction D. It is probable therefore that the furfural comes from ketonic material. Of the NaOI-resistant material a small amount, representing in most urines less than 1.0 mg./4 hr., can be accounted for by the presence of small amounts of fructose, as revealed by mycological analysis. A considerable portion of the remainder, over 2 mg./4 hr. as xylose, is furfural-producing material.

It would appear probable that the reducing material destroyed by the NaOI would be similarly affected by Br oxidation after 9–16 hr. Br treatment however causes an increase in the reducing power of, or the production of new reducing substances from, certain components of the urine fractions. This is quite apparent when the curve produced by Fraction B is compared with that from Fraction D, where all the free aldose has been destroyed by NaOI. In Fraction B there is a rapid drop in reducing power followed by a steady rise but in Fraction D no drop occurs, but only a rise which is very rapid in the first few hours and which reaches completion at the end of 8 days. Everett *et al.* [1934, 1] have ascribed the rise in the reducing value of whole urine produced by bromination to acid hydrolysis, but this could not be an important factor in the urine fractions studied here because no decrease in pH occurred and the rise in Cu reducing value took place in Fraction F where the sugars released by vigorous acid hydrolysis had been destroyed. It should also be noted that all the substances liberated by such acid hydrolysis acted as aldose towards NaOI and Br, so that if acid hydrolysis were to occur during bromination the products would be promptly destroyed. It has also been observed that with lactose and maltose, and also in the case of acid-hydrolysable urine sugar, both Br and NaOI oxidize the free reducing groups and subsequent acid hydrolysis liberates the protected reducing group. In the case of maltose and lactose such reducing sugar is liberated in theoretical amounts by acid hydrolysis after Br oxidation and is identified by mycological analysis as glucose and galactose respectively. Sucrose is not affected by either Br or NaOI.

It seems that during bromination one or probably more reducing substances resistant to oxidation by Br are formed or liberated, either by the action of Br on some substance which previously had no reducing value or by an increase in the reducing power of some substance originally only feebly reducing. The reducing value is increased to approximately  $2\frac{1}{2}$  times the reduction due to substances left after NaOI, as can be seen by examination of the Br oxidation curve of Fraction D, in which all the free aldose has been destroyed by NaOI. Comparison of Fractions D and F shows that there are two reactions tending to increase the reducing substances, one which is relatively rapid and which is almost completed in 24 hr. and one which is much slower and which is not

complete until after 1 week. The second reaction does not take place in Fraction F. That is, hydrolysis permits the NaOI to destroy the substance which on Br treatment gives rise to a slow increase in reducing power. This, and the fact that acid hydrolysis (Fraction C) does not give rise to this substance would indicate that the increase in reducing power is more likely to be due to the production of a new compound than to the liberation of one already present.

Presumably these reactions are taking place in Fractions B, C and E during the first 6 hr. of Br treatment, but their effect is obscured by the much more rapid destruction of aldose. The shape of the rising curve after 12 hr. suggests that by the extension of this latter part back to zero time the curve would fall in the case of Fraction B near the starting-point of D, and in the case of C, near that of F. It is probable that a considerable part of the initial rise observed by Everett *et al.* in whole urines is due to the same compound which causes the rise in reduction in Fractions D and F and that this latter reaction is not caused by acid hydrolysis. That an initial rise does not occur in cleared urines is due at least partly to the fact that such solutions are much more readily saturated with Br than are whole urines, which contain large amounts of halogen-absorbing materials. Whereas the increase in copper-reducing ability of the Br-resistant fraction is almost as rapid in low concentration of Br as in a saturated solution, the oxidation rate of aldoses is affected greatly by Br concentration. Similar increases in reducing power were observed in filtrates from individual urines cleared with  $\text{HgSO}_4$ -KOH according to the directions of Everett *et al.* [1934, 2]. An extreme example of this is shown in Fig. 1. Other samples gave much smaller rises and a few gave curves similar to those described by Everett *et al.* [1934, 1].

As in the case of NaOI, Br oxidation leaves appreciable amounts of furfural-producing material.

It is evident that NaOI and Br oxidations are not of equal value in determining the ratio of aldose to non-aldose reducing substances in urine, but that owing to the fact that Br treatment gives rise to new reducing substances the latter type of analysis may give a false impression of the amount of non-oxidizable material. It would appear probable that the Br-resistant reducing material termed by Everett *et al.* [1934, 1] uroketose is not a single substance but is composed, in so far as those portions which are precipitated by copper-lime are concerned, of (a) Br- and NaOI-resistant reducing substances already present in urine and (b) new reducing material produced by the action of Br. It is probable that a part at least of (a) is a ketopentose, as evidenced by the production of furfural, considered with the action of the pentose-removing yeasts.

#### SUMMARY

1. By the use of basic Pb acetate,  $\text{HgSO}_4$  and a copper-lime precipitation method, a fraction of urine free from N and containing a high percentage of true sugars was obtained.

2. The changes in reducing power induced by NaOI and Br oxidations were observed.

3. Br oxidation gives rise to the production of new reducing substances resistant to further Br and NaOI oxidation and to yeast fermentation.

4. NaOI oxidation gives rise to little of any such substance and shows that 75% of the reducing material behaves as does aldose.

5. Evidence is presented which suggests the presence of small amounts of pentose in urine, some of which is ketonic in nature.

The writers wish to express their gratitude to the late Prof. V. J. Harding for helpful suggestions and criticism during the early stages of the work.

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# LXIV. THE FATE OF CALCIUM AND MAGNESIUM AFTER INTRAVENOUS ADMINISTRATION TO NORMAL PERSONS

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FIFTEEN years ago there would seem to have been no doubt in the minds of physiologists that the divalent and trivalent metals were excreted mainly by the gastro-intestinal tract and that variations in the rate of this excretion maintained the normal levels within the body. This is still the opinion of most people who have not studied the subject, but within the last 10 or 12 years evidence has been collected which makes it essential to reconsider the whole question.

First, it has been shown that iron is certainly excreted much less freely than was previously supposed [McCance & Widdowson, 1937, 1]. It is, in fact, impossible to demonstrate any gastro-intestinal excretion of iron by man [Widdowson & McCance, 1937; McCance & Widdowson, 1938], although it is agreed that traces of this metal must get into the intestine from abraded cells and glandular secretions. It has, therefore, been suggested that the amount of iron in the body must be regulated by controlled absorption [McCance & Widdowson, 1937, 2] and this seems to have been supported by the most recent evidence [Hahn *et al.* 1938]. Secondly, much recent work on Ca has tended to show that in normal circumstances its excretion into the gastro-intestinal tract is negligible or at least plays no part in regulating the amount in the body [Christiansen, 1936; Aub *et al.* 1937; Henry & Kon, 1939]. The best evidence has been obtained (a) by the direct determination of the Ca excreted into the isolated large intestines of dogs [Nicolaysen, 1934] and men [Welch *et al.* 1936; Johnson, 1937], (b) by the determination of Ca balances under experimental conditions usually involving the parenteral administration of the metal [Nicolaysen, 1934; Christiansen, 1936; Albright & Sulkowitch, 1938]. Against this view, however, must be set certain rather older experiments such as those of Greenwald & Gross [1925] on dogs, Ross & Scriver [1928] and Pugsley [1932] on rats. From a study of faecal pellets Cowell [1937] also considers that rabbits excrete Ca into their large intestines. This excretion may be peculiar to the rabbit and in any case it probably has no regulatory function [Christiansen, 1936]. If the balance experiments which have been carried out on normal persons and on hyperthyroid patients are correct, relatively large quantities of Ca may be passed into the human gastro-intestinal tract, even when the food contains very little [Bauer *et al.* 1929; Tibbetts *et al.* 1932; Tibbetts & Aub, 1937, 2]. There is nothing to suggest however that this excretion has any regulatory function, but nevertheless, it requires further investigation. Thirdly, what little work has been carried out on Mg has also tended to show that this metal is not excreted in more than very small quantities into the gastro-intestinal tract [Ferguson & Ivy 1936; Nicolaysen, 1936].

In contrast to this it still appears true to say that certain non-physiological heavy metals are excreted predominantly by the gastro-intestinal tract. Thus,

lead and radium have been shown to be so excreted when they have been given parenterally or when they have been mobilized from the bones by parathormone [Hunter & Aub, 1926-7; Schlundt & Failla, 1931; Aub, 1935; Aub *et al.* 1938]. Mercury has been found to be excreted—slightly at any rate—in the faeces after parenteral administration [Young *et al.* 1930]. The site of the excretion of these metals, however, is not yet known. Histological attempts have been made to solve the problem [Schönlebe, 1937], but physiological experiments should be undertaken, similar to those which have already been carried out for Ca and Fe on animals with isolated large intestines.

The experiments now to be described were undertaken in conjunction with work on the excretion of Fe [Widdowson & McCance, 1937; McCance & Widdowson, 1938]. They are, it is believed, the first of their kind to be made on strictly normal persons, and the results are in keeping with the view that the amount of Ca or of Mg in the body is not regulated by glands which secrete into the gastrointestinal tract.

#### EXPERIMENTAL ARRANGEMENT AND TECHNIQUE

The work was carried out at King's College Hospital, London, on the same six persons already described [McCance & Widdowson, 1938]. Balance experiments were performed at two different (physiological) levels of intake by mouth and finally during a time of parenteral administration. Each balance experiment lasted 14 days and was preceded by a preliminary period of 2 or 3 days. The dietary regime was continued beyond the 14 days until the last specimen of faeces had been collected. Distilled water was used for drinking purposes and in the preparation of beverages. The use of toothpaste was avoided. The increased oral intake of Ca was brought about by increasing the intake of milk and that of Mg by administering medicinal supplements. Both metals were injected intravenously as the gluconates; the method of so doing was described by McCance & Widdowson [1938]. 0.186 g. of Ca and 0.219 g. of Mg were given daily. Even run in slowly over a period of 30-45 min. the intravenous injection of these amounts to normal persons is rather an uncomfortable experience, for it is accompanied by constant flushings and sometimes by nausea, but no further difficulties were encountered except that one subject (R. M. L.) fainted at the close of his 7th injection. The management of the food, drinks, urine and faeces has been described by McCance & Widdowson [1938] and the chemical methods employed were those described by McCance *et al.* [1936].

#### RESULTS

##### (1) *Calcium*

Table I shows the results for the six subjects at the lower levels of intake which varied from 6.43 to 9.20 g. Ca in the 14 days—an average of 0.582 g. Ca/subject/day. R. A. M. and R. H. E.'s balances were rather unaccountably negative but the summarized results amounted to a negative balance of only 2.03 g. on an intake of 49 g.

Table II gives the results at the higher levels of intake by mouth which now varied from 8.10 to 12.88 g. Ca in the 14 days and averaged 0.734 g. Ca/subject/day. R. A. M. and R. H. E. were still in negative balance and it should be pointed out that these negative balances cannot be explained by incomplete collections of faeces for at this stage both subjects were in positive iron balance [McCance & Widdowson, 1938]. The summarized results showed the group to be practically in balance.

Table I. *Ca balances at the lower levels of intake*

Subject	Ca intake from food, g.	Urine	Ca output, g.		Ca balance, g.
			Faeces	Total	
E. M. W.	9.20	1.34	7.50	8.84	+ 0.36
M. M.	8.61	2.20	6.86	9.06	- 0.45
H. L.	6.43	0.64	5.72	6.36	+ 0.07
R. A. M.	7.82	2.49	6.37	8.86	- 1.04
R. M. L.	8.84	2.81	5.75	8.56	+ 0.28
R. H. E.	7.98	2.50	6.73	9.23	- 1.25
Totals	48.88	11.98	38.93	50.91	- 2.03

Table II. *Ca balances at the higher levels of intake*

Subject	Ca intake from food, g.	Ca output, g.			Ca balance, g.
		Urine	Faeces	Total	
E. M. W.	11.35	1.70	9.81	11.51	- 0.16
M. M.	11.92	2.47	9.40	11.87	+ 0.05
H. L.	8.10	0.87	7.10	7.97	+ 0.13
R. A. M.	8.97	2.42	7.10	9.52	- 0.55
R. M. L.	12.88	3.53	9.12	12.65	+ 0.23
R. H. E.	8.46	2.70	6.46	9.16	- 0.70
Totals	61.68	13.69	48.99	62.68	- 1.00

Table III contains the results when Ca was administered intravenously. The oral intakes varied from 7.09 to 9.78 g. Ca in the 14 days—an average of 0.628 g. Ca/subject/day. Neglecting for the moment individual results, it is evident that additional Ca, equivalent to a very great part if not the whole of the Ca administered intravenously, had been excreted before the experiment came to an end, since the summarized results for the group indicated a positive balance of only 2.52 g. on an intake of 68.31 g. Unfortunately it is not possible to define the limits of error in an experiment of this kind, but from the individual results it would appear that R. M. L. and possibly R. H. E. had not excreted as much additional Ca as had been injected into them by the end of the experiment—but that the others had certainly done so.

Table III. *Ca balances during intravenous administration of the metal*

Subject	Ca intake, g.			Ca output, g.			Ca balance g.
	From food	From injection	Total	Urine	Faeces	Total	
E. M. W.	8.54	2.60	11.14	3.37	7.37	10.74	+ 0.40
M. M.	9.00	2.60	11.60	5.04	6.59	11.63	- 0.03
H. L.	7.09	2.60	9.69	2.74	6.27	9.01	+ 0.68
R. A. M.	9.78	2.60	12.38	5.21	8.11	13.32	- 0.94
R. M. L.	9.40	2.60	12.00	4.75	5.92	10.67	+ 1.33
R. H. E.	8.90	2.60	11.50	4.17	6.25	10.42	+ 1.08
Totals	52.71	15.60	68.31	25.28	40.51	65.79	+ 2.52

Table IV shows the ratios of urinary/faecal Ca and faecal/food Ca of all the subjects during each stage of the experiment. The relative constancy of the urinary/faecal ratio at both levels of intake by mouth suggests that during the period of high Ca intake the metal was divided between the urine and faeces in about the same proportions as it had been at the lower level of intake. The large increase in the ratio during intravenous administration indicates that in these circumstances most of the additional Ca was being excreted in the urine. In

Table IV

Subject	Ratios Urinary Ca/Faecal Ca			Ratios Faecal Ca/Food Ca		
	Lower intakes	Higher intakes	Injection stage	Lower intakes	Higher intakes	Injection stage
E. M. W.	0.18	0.17	0.46	0.82	0.87	0.86
M. M.	0.32	0.26	0.77	0.80	0.79	0.73
H. L.	0.11	0.12	0.44	0.89	0.88	0.88
R. A. M.	0.39	0.34	0.64	0.81	0.79	0.83
R. M. L.	0.49	0.39	0.80	0.65	0.71	0.63
R. H. E.	0.37	0.42	0.67	0.84	0.76	0.70
Average	0.31	0.28	0.63	0.80	0.80	0.77

fact, since there was no rise in the faecal/food Ca ratios during this time, it is clear that all the additional Ca, equivalent to the whole or almost the whole of the amount injected, must have been excreted by the kidney.

It is possibly of some interest to note that although the urinary/faecal Ca ratio is remarkably constant in any one individual it varies considerably from one individual to another. The findings of Owen [1939] in a study of 10 adult and elderly males were very much the same. Thus about 30 % of R. M. L.'s total output of Ca was found in the urine, whereas only about 10 % of H. L.'s. It would be an interesting problem to find out why this should be so. In the light of the experiments in which Ca was given intravenously, it would look as though H. L. consistently absorbed less Ca than did R. M. L. It may further be noted that the three female subjects (E. M. W., H. L., and M. M.) had the three lowest urinary/faecal Ca ratios, but it is impossible to say whether this represents a real sex difference since the number of subjects was so small. The work of Owen [1939] suggests that occasionally males may excrete as little as 10–11 % of their total Ca in the urine.

## (2) Magnesium

Table V shows the results for the six subjects at the lower levels of intake, which varied from 3.21 to 4.44 g. Mg in the 14 days, an average of 0.271 g. Mg/person/day. On those amounts of Mg all the subjects were in fair balance and the summarized results amounted to an insignificant positive balance of 0.22 g. on an intake of 22.8 g.

Table V. *Mg balances at the lower levels of intake*

Subject	Mg intake from food, g.	Mg output, g.			Mg balance, g.
		Urine	Faeces	Total	
E. M. W.	3.40	1.51	2.25	3.76	- 0.36
M. M.	3.87	1.44	2.36	3.80	+ 0.07
H. L.	3.21	1.07	2.10	3.17	+ 0.04
R. A. M.	4.44	1.74	2.52	4.26	+ 0.18
R. M. L.	3.98	1.81	1.95	3.76	+ 0.22
R. H. E.	3.90	1.71	2.12	3.83	+ 0.07
Totals	22.80	9.28	13.30	22.58	+ 0.22

Table VI indicates the results on the higher levels of intake by mouth, which varied from 5.18 to 6.38 g. Mg in the 14 days and averaged 0.405 g. Mg/subject/day. All the subjects were again in very fair balance and the summarized results again amounted to a small positive balance of 0.73 g. on a total intake of 34 g.

Table VI. *Mg balances at higher levels of intake*

Subject	Mg intake, g.			Mg output, g.			Mg balance g.
	From food	From medicine	Total	Urine	Faeces	Total	
E. M. W.	3.51	1.76	5.27	1.71	3.41	5.12	+ 0.15
M. M.	3.57	1.76	5.33	1.18	3.88	5.06	+ 0.27
H. L.	2.78	2.40	5.18	1.38	3.84	5.22	- 0.04
R. A. M.	3.98	2.40	6.38	1.85	4.23	6.08	+ 0.30
R. H. L.	3.79	1.76	5.55	2.18	3.45	5.63	- 0.08
R. H. E.	3.90	2.40	6.30	2.18	3.99	6.17	+ 0.13
Totals	21.53	12.48	34.01	10.48	22.80	33.28	+ 0.73

Table VII gives the results when Mg was administered intravenously. The oral intakes ranged from 3.20 to 4.28 g. in the 14 days, and averaged 0.279 g. Mg/subject/day. 3.06 g. Mg were given intravenously to each subject (0.219 g./day). It is evident from the balances that, as with Ca, additional Mg equal to practically the whole of the quantity given intravenously had been excreted before the experiment terminated. The summarized results showed a positive balance of 2.19 g. on a total intake of 41.78 g. The individual results suggest that M. M. was the only subject who had not got rid of the injected Mg.

Table VII. *Mg balances during the intravenous administration of the metal*

Subject	Mg intake, g.			Mg output, g.			Mg balance g.
	From food	From injection	Total	Urine	Faeces	Total	
E. M. W.	3.75	3.06	6.81	4.53	2.10	6.63	+ 0.18
M. M.	3.79	3.06	6.85	3.39	2.15	5.54	+ 1.31
H. L.	3.20	3.06	6.26	3.77	2.21	5.98	+ 0.28
R. A. M.	4.14	3.06	7.20	3.98	2.47	6.45	+ 0.75
R. M. L.	4.26	3.06	7.32	4.75	3.16	7.91	- 0.59
R. H. E.	4.28	3.06	7.34	4.77	2.31	7.08	+ 0.26
Totals	23.42	18.36	41.78	25.19	14.40	39.59	+ 2.19

Table VIII

Subject	Ratios Urinary Mg/Faecal Mg			Ratios Faecal Mg/Ingested Mg		
	Lower intakes	Higher intakes	Injection stage	Lower intakes	Higher intakes	Injection stage
E. M. W.	0.67	0.50	2.16	0.66	0.65	0.56
M. M.	0.61	0.30	1.58	0.61	0.73	0.57
H. L.	0.51	0.36	1.70	0.65	0.74	0.69
R. A. M.	0.69	0.44	1.62	0.57	0.66	0.60
R. M. L.	0.93	0.63	1.50	0.49	0.62	0.74
R. H. E.	0.81	0.55	2.07	0.54	0.63	0.54
Average	0.70	0.46	1.77	0.58	0.67	0.62

Table VIII gives the ratios of urinary/faecal Mg and faecal/food Mg of all the subjects during each stage of the experiment. The fall in the urinary/faecal ratio during the higher oral intakes indicates that excretion of the additional Mg was not shared in the previous proportions between the urine and faeces. Tibbets & Aub [1937, 1] also found this to happen but their Mg supplements were much larger. Indeed, a comparison of the summarized results of Tables V and VI suggests that the kidney excreted very little of the extra Mg taken by mouth, and in this respect the results differ from those obtained with Ca. In all probability very little of the extra Mg taken by mouth was absorbed. The large increase



in the urinary/faecal ratio during the fortnight of intravenous administration indicates that, as in the case of Ca, the kidney was excreting much of the extra Mg, and a study of the food/faecal ratios shows that, as with Ca, none of the additional Mg was finding its way into the faeces.

### DISCUSSION

These experiments are only a small contribution towards the solution of a very large and complicated problem, but they raise interesting points which deserve consideration. They would seem to show, first, that the injection of Ca or Mg intravenously into normal persons provokes no increased excretion of these metals by the gastro-intestinal tract, and taken in conjunction with previous work they support the view put forward earlier in this paper that the gastro-intestinal tract does not excrete these metals in amounts which vary with the levels in the plasma or with the needs of the body. It would be very interesting to be able to say why lead and radium are treated so differently. Secondly, these experiments show quite clearly that Ca and Mg given intravenously to normal persons are rapidly excreted by the kidney, and it follows that the normal kidney must be exceedingly sensitive to variations in the levels of these metals in the serum. This mechanism must always be in operation and should be seriously considered whenever variations in the urinary excretion of these metals have to be explained. It would seem from the literature, however, that in hyperthyroidism and sometimes in hyperparathyroidism there may be a large increase in the output of Ca in the urine without a corresponding increase in the serum Ca, so that other factors are probably involved, at any rate in the case of this metal. In the absence of an acidosis in these diseases, which might be expected to raise the urinary excretion of these metals by increasing the concentration of ultra-filterable ions in the serum, this must be due to a change in the activity of the kidney itself. It would be most interesting and valuable to know in what way secretions of the thyroid and parathyroid affected the kidney.

### SUMMARY

1. 0.186 g. Ca and 0.219 g. Mg were injected intravenously into six normal persons every day for a fortnight.
2. This led to a rapid increase in the excretion of Ca and Mg by the kidney and in most subjects the additional output was equal to the amount injected.
3. No evidence was found that any additional Ca or Mg was excreted into the gastro-intestinal tract.

The authors very much appreciated the co-operation of their fellow subjects, and of Mrs Ellis who was responsible for much of the dietary side of the experiment. The Medical Research Council paid a large part of the expenses and E. M. W. is indebted to the Council for a personal grant. The gluconates were obtained from "Sandoz" Products, Ltd., and the magnesium salt was very kindly presented by the Company free of charge.

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## LXV. ANALYSIS OF PROTEINS

### XI. THE PRODUCTS OF THE ACTION OF SODIUM HYDROXIDE ON CASEINOGEN. THE COMPOSITION OF DEPHOSPHOCASEIN, OR DEPOCASEIN

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PLIMMER & BAYLISS [1906] first showed that the P of caseinogen was completely separated as phosphoric acid by the action of 1 % NaOH at 37° in 24 hr. They also noticed that the solution became very milky in appearance and that acidification precipitated a substance resembling caseinogen. Rimington & Kay [1926] confirmed these and other observations of Plimmer & Bayliss and called the substance thrown down by acetic acid "dephosphorized caseinogen", although it represented only about 40 % of the caseinogen. Rimington [1927] analysed dephosphorized caseinogen by the Van Slyke method and noticed little difference in composition from that of caseinogen except in amide-N. No other work upon this substance has since been published. Meanwhile new and improved methods of analysis of proteins have been devised, and they have now been used in further examination of this substance. Though our results are incomplete, this substance has resemblances with caseinogen, but must be considered different. The term "dephosphorized caseinogen" chosen by Rimington & Kay is not altogether appropriate since caseinogen is converted by 1 % NaOH into this product and a substance or mixture of substances resembling a primary proteose in about equal parts. The latter is a caseose and as it does not contain P is a dephosphocaseose, or shortly depocaseose. The former resembles a meta-protein in solubilities in 0.1 *N* alkali and acid and caseinogen in being precipitated by more acid. It might be called metacasein. This term does not indicate the absence of phosphorus. It is a dephosphometacasein, or for brevity dephosphocasein or depocasein.

It was hoped that chemical examination of the intermediate products of hydrolysis of proteins would show a less complex mixture of amino-acids, so that ultimately it might be possible to fill in the gaps in the amino-acid composition and find out in which order the amino-acids were combined. In the case of caseinogen it appears possible that the whole molecule is made up of two or more very similar portions. Sørensen [1930] adopted the view that caseinogen consisted of many similar molecules of the same solubility.

#### EXPERIMENTAL

##### *Light white soluble casein. L.W.S. casein*

Light white soluble casein supplied by The British Drug Houses, Ltd., has been used for the preparation of depocasein. Dr F. H. Carr kindly informed us that this material is the sodium salt of caseinogen and is prepared by allowing fat-free milk to develop lactic acidity and then precipitating caseinogen by the

addition of very dilute HCl. The caseinogen is washed with water and converted into the sodium salt. Two separate batches supplied at different times gave the following percentage analyses:

Batch	H <sub>2</sub> O lost at 105°	N	P	S	Ash Na	Corrected for H <sub>2</sub> O and Na contents		
						N	P	S
1	9.9	12.6	0.72	0.42	3.9 - 1.6	14.2	0.81	0.47
2	10.1	12.5	0.67	0.59	3.5 - 1.4	14.1	0.76	0.66

The computation of the amount of Na in the ash is based upon the figures for P and S as follows:

0.72 % P corresponds with 3.7 % Na <sub>3</sub> PO <sub>4</sub>	or 3.0 % Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub>	or 2.3 % NaPO <sub>3</sub>
containing Na = 1.56 %	or 1.04 %	or 0.52 %
and leaving Na <sub>2</sub> CO <sub>3</sub> - 0.2	or 0.9	or 1.56
containing Na = 0.09	or 0.39	or 0.7
Total Na in ash = 1.65	or 1.43	or 1.22
0.42 % S corresponds with 1.78 % Na <sub>2</sub> SO <sub>4</sub>	containing	0.58 % Na
Remainder of ash as Na <sub>2</sub> CO <sub>3</sub> 2.12 %	containing	0.92 % Na
Total Na --	—	1.5 %

If the ash were pure Na<sub>2</sub>CO<sub>3</sub> - 3.9%, it would contain 1.70% Na. The Na content of the L.W.S. casein is thus about 1.6%; similarly for batch 2 with 3.5% ash, the Na is about 1.4%.

#### Preparation of depocasein

The conditions for precipitating depocasein from the alkaline digest of caseinogen were not mentioned by Plimmer & Bayliss. Rimington & Kay simply stated that acetic acid was added to give maximum precipitation. In order to determine the optimum amount of acetic acid for precipitation 5 g. of L.W.S. casein were dissolved in water, 62.5 ml. of *N* NaOH added, the solution brought to 250 ml. and placed in an incubator at 37°. After 24 hr. the solution was cooled till it occupied the original volume. 10 ml. were removed to determine the total N, and five lots of 25 ml. were placed in Kjeldahl digestion flasks. Varying volumes of 25 % v/v. (4.32 *N*) acetic acid were added to precipitate the depocasein. The sticky precipitates adhered to the bottom and sides of the flasks. After 24 hr. the contents of each flask were filtered and the precipitates washed with 20 ml. of 0.5 % acetic acid. The filters were returned to their respective flasks and N determinations carried out with the following results:

Total N of the digested solution - 2.49 g./l.						
ml. acetic acid added to 25 ml.	4.20	4.30	4.40	4.50	4.60	4.70
N pptd. in g./l.	1.28	1.32	1.36	1.30	1.29	1.28

The maximum yield of depocasein was thus obtained with 4.40 ml. and the precipitate contained  $\frac{1.36 \times 100}{2.49} = 55\%$  of the total N.

Depocasein was prepared in large quantities by dissolving 40 g. L.W.S. casein by grinding with water, placing in a Winchester bottle, adding 500 ml. *N* NaOH, diluting to 2 l., warming to 37° and maintaining at this temperature for 24 hr. The solution was then transferred to a large beaker and 87 ml. of glacial acetic acid run in from a burette during 5-10 min. with constant stirring. Most of the precipitate adhered to the beaker; the solution was poured off after 24 hr. and subsequent precipitations carried out on top of the first. When sufficient had been collected, usually from four bottles, the precipitates were re-dissolved by grinding with cold *N* NaOH and immediately reprecipitated with dilute H<sub>2</sub>SO<sub>4</sub>, the acid being added until the precipitate aggregated and the mother liquor gave no further precipitate with acid. The depocasein was filtered, washed with water and dried with successive changes of absolute alcohol and

finally in a vacuum desiccator over  $\text{H}_2\text{SO}_4$ . The product was a fine white powder and the yield approximately 40 % of the L.W.S. casein taken, some having been lost during purification. Two separate batches of about 250 and 500 g. gave the following percentage analytical figures:

Batch	$\text{H}_2\text{O}$	N	P	S	Ash	Na	Corrected for $\text{H}_2\text{O}$ and Na		
							N	P	S
1	19.7	10.90	0.025	0.34	0.7	0.3	13.6	0.03	0.43
2	10.1	11.97	0.030	1.08	0.7	0.3	13.4	0.03	1.20

The S content of batch 2 is erroneous. Analyses of further batches of depocasein by Mr T. J. R. Macara showed 0.42 and 0.45 % S. The low P content compares with the similar figure of Rimington & Kay. The N content is lower than that of caseinogen owing to removal of amide-N by the action of NaOH.

Depocasein dissolves easily in 0.1 N NaOH and 0.1 N HCl and is precipitated on neutralization and by addition of 2 N HCl. The solution in 0.1 N alkali made just neutral to incipient precipitation with 0.1 N  $\text{H}_2\text{SO}_4$  gives gelatinous precipitates with silver nitrate, lead acetate, copper sulphate and ferric chloride solutions. With mercuric chloride there was no precipitate till 2 N HCl was added. Mercurous nitrate gave a white precipitate which rapidly became black. Calcium chloride, barium chloride and magnesium sulphate solutions did not give precipitates.

The filtrate from depocasein is almost completely precipitated by half saturation with ammonium sulphate, but appears to contain a mixture of proteoses. On evaporating a volume of filtrate containing 15.8 g. N *in vacuo* at 45°, by which some of the acetic acid was removed, a syrup containing sodium acetate remained. The crystals dissolved on diluting with water leaving an insoluble gelatinous mass. The liquid was poured off and the mass washed with water and allowed to dry. It was found to be insoluble in water and alcohol, but soluble in a range of mixtures of water and alcohol. It weighed 28 g. and contained 10.8 % N. The liquid after concentration was fractionated with alcohol. The first fraction weighing 2 g. was insoluble in water. A second fraction of 14.5 g. was thrown out at about 60 % concentration of alcohol and a third fraction of 8.9 g. at over 90 % alcohol. Both the latter were soluble in water and the third fraction was hygroscopic.

#### Distribution of N

L.W.S. casein, depocasein, its filtrate and the three fractions, also a depocasein made after 144 hr. treatment with 1 % NaOH were examined for distribution of N by Van Slyke's method by the procedure described by Plimmer & Rosedale [1925] using 3 g. (2 g. of fraction 1) of protein in each analysis. The results, the mean of two determinations, are shown in the following table together with the data of Rimington & Kay (R. & K.) for dephosphorized caseinogen:

	L.W.S. casein		Depocasein		Filtrate	Fractions			144-hr. depocasein
	Batch 1	Batch 2				1	2	3	
Amide-N	11.1	11.6	5.3	4.4	1.9	4.4	7.1	5.5	0.2
Humin-N	1.2	2.0	0.9	1.8	3.2	1.6	1.2	1.7	0.1
Arginine-N	8.0	11.6	8.8	6.8	7.4	7.5	6.9	9.0	9.0
Histidine-N	5.1	2.3	8.8	7.0	8.2	10.4	0	4.9	6.3
Lysine-N	7.9	11.9	9.8	13.4	8.8	9.1	13.8	11.0	14.7
Monoamino:									
Amino-N	60.0	56.8	65.0	60.2	49.0	60.7	65.4	67.9	65.8
Non-amino-N	2.5	4.0	0	3.9	14.3	5.7	5.0	0	0.7
Total N recovery	101.8	100.2	98.6	97.5	92.8	99.4	99.4	100.0	96.8
Total N of solid	14.2	14.1	13.6	—	—	10.8	11.4	6.8	—

In the first place our figures do not entirely agree with those of Rimington & Kay. The most noticeable differences are those for lysine-N and the non-amino-N of the monoamino fraction. Our figure is erroneous as proline has actually been isolated (below). The absence or low content of non-amino-N in depocasein is accounted for by its high value in the filtrate, but the fractions isolated did not have such high amounts.

The amide-N of the filtrate is after determination of ammonium salt by distillation *in vacuo* with baryta or sodium carbonate and alcohol and recalculation to a total N of  $100 - 19 = 81\%$  of the original total N of the filtrate.

Of the bases, arginine-N is about the same in L.W.S. casein, depocasein and the filtrate. Histidine appears to be greater in depocasein than in L.W.S. casein. The lysine-N, which is dependent on the accuracy of the determination of the other bases, is variable. There is unaccountable variation in the figures of the monoamino fractions.

#### *Determination of arginine, histidine and lysine*

Three separate analyses of both L.W.S. casein and depocasein for the amounts of the three basic amino-acids were made by the improved methods of Vickery and his co-workers as described in several separate papers [1926-33]. A final description of the best procedure was not published. The procedure adopted in these analyses was briefly the following.

*Hydrolysis.* 50 g. of protein were hydrolysed with 240 ml. conc. HCl and 160 ml. water for 30 hr. HCl was partly removed by three evaporations *in vacuo* and then completely by precipitation with  $\text{Ag}_2\text{O}$  and  $\text{H}_2\text{SO}_4$  at  $\text{pH} < 2.8$ . The  $\text{AgCl}$  was extracted with dilute HCl and then with water, and chlorides again removed from the extract. The chloride-free solution and extracts were evaporated, brought to 2 l. and total N estimated in 10 ml. to ascertain the amount of protein present.

*Precipitation of histidine and arginine.* After concentrating to 1 l. excess of conc.  $\text{AgNO}_3$  solution was added, as shown by testing a portion with baryta solution, and hot baryta solution added to  $\text{pH} 12$  (alizarin blue S). The silver precipitate of arginine and histidine was filtered off next day, and without washing suspended in water, adjusted to  $\text{pH} 2.8$  with  $\text{H}_2\text{SO}_4$  and decomposed with  $\text{H}_2\text{S}$ . The filtrate and washings from  $\text{Ag}_2\text{S}$  were evaporated *in vacuo*, brought to 500 ml. and N determined on 10 ml.

*Histidine.* Histidine silver was precipitated by adding  $\text{Ag}_2\text{O}$  and  $\text{H}_2\text{SO}_4$  alternately until excess of silver was present keeping the reaction below  $\text{pH} 2.8$  and then cautiously adding cold baryta solution until the reaction was between  $\text{pH} 7.2$  and  $7.8$ , as shown by the greenish colour of a drop of bromophenol blue on the surface and flocculation of histidine silver. The precipitate was again dissolved and thrown out at a reaction as near as possible to  $\text{pH} 7.4$ . The histidine silver was filtered off next day and washed once with water. It was suspended in water, made acid with  $\text{H}_2\text{SO}_4$  and decomposed with  $\text{H}_2\text{S}$ . The solution was evaporated and brought to 250 ml. 5 ml. were used for N estimation and the remainder treated with an equal volume of 10 %  $\text{HgSO}_4$  in 2N  $\text{H}_2\text{SO}_4$ . The precipitate of histidine mercuric sulphate after washing with 2.5 %  $\text{HgSO}_4$  in N  $\text{H}_2\text{SO}_4$  was suspended in water and decomposed with  $\text{H}_2\text{S}$ . The solution and washings were evaporated and brought to 500 ml. N was determined in 5 ml. and the remainder concentrated and treated with a slight excess of flavianic acid (14.96 g. for 1 g. histidine N). Histidine diflavianate crystallized out and a

further crop was obtained from the filtrate. Both crops were recrystallized, the pure crystals dried at  $100^{\circ}$  and weighed. They had a decomposition point of  $235^{\circ}$ .

*Arginine.* The filtrates from the two precipitations of histidine silver were combined, acidified with  $\text{H}_2\text{SO}_4$ , evaporated *in vacuo*, brought to 1 l. and N determined in 20 ml. The remainder, tested for excess of Ag which was present, was treated with hot baryta solution to pH 12. The precipitate of arginine silver was filtered off, washed twice with baryta solution, suspended in water, acidified with  $\text{H}_2\text{SO}_4$  and decomposed with  $\text{H}_2\text{S}$ . The solution and washings from  $\text{Ag}_2\text{S}$  were concentrated, brought to 250 ml. and arginine-N estimated in 5 ml. To the remainder, heated to boiling, the requisite amount of flavianic acid (5.61 g. for 1 g. arginine-N) in hot water was added. Arginine monoflavianate crystallized out and a further crop of diflavianate was obtained from the filtrate. The two filtrates at pH 12 were combined, acidified, evaporated to a small volume and precipitation at pH 12 repeated. No further precipitation occurred showing that no arginine had escaped the first precipitation.

*Lysine.* The filtrates from the two precipitations at pH 12 containing lysine and other amino-acids, also baryta and silver, were acidified with  $\text{H}_2\text{SO}_4$ , saturated with  $\text{H}_2\text{S}$  and the precipitate removed and washed. The solution was neutralized with NaOH to Congo red and evaporated *in vacuo*. Ammonia was removed by making alkaline to phenolphthalein, adding 2 vol. alcohol and distilling *in vacuo*. After acidifying to pH 2.8, making to 500 ml. and estimating N in 10 ml., the remainder was acidified with  $\text{H}_2\text{SO}_4$  to 5 % by weight and precipitated with 20 % phosphotungstic acid. The lysine phosphotungstate after 4 days in the dark was filtered off and washed with 2.5 % phosphotungstic acid in 5 %  $\text{H}_2\text{SO}_4$ , dissolved in a mixture of acetone and water and decomposed with hot baryta solution until the reaction was permanently alkaline to phenolphthalein. The filtrate and washings from Ba phosphotungstate were acidified with  $\text{H}_2\text{SO}_4$ , filtered from  $\text{BaSO}_4$ , concentrated and reprecipitated with phosphotungstic acid as before at a volume of 650 ml. After 24 hr. the lysine phosphotungstate was filtered off and recrystallized from 5 %  $\text{H}_2\text{SO}_4$  containing 2.5 % phosphotungstic acid. After 4 days the crystalline lysine phosphotungstate was filtered off and decomposed as above with acetone and baryta. Acetone was removed by evaporation and Ba quantitatively with  $\text{H}_2\text{SO}_4$ . The solution and washings were brought to 250 ml. and N determined in 5 ml. The remainder was concentrated *in vacuo* to a thin syrup, alcohol added till slight precipitation occurred and saturated alcoholic picric acid solution carefully added till lysine picrate was completely thrown down. The precipitate was washed with alcohol and ether, dried in air and weighed.

With allowances made for aliquots removed for N determinations and for solubilities of histidine diflavianate, arginine monoflavianate and lysine picrate the quantities obtained were:

	L.W.S. casein			Depocasein		
	(1)	(2)	(3)	(1)	(2)	(3)
Arginine flavianate	3.20	3.75	3.91	3.20	3.20	3.73
Histidine diflavianate	0.97	2.71	3.46	2.07	3.23	2.66
Lysine picrate	3.84	Lost	4.52	4.49	3.03	3.44

The results of the analyses were thus variable and disappointing. In one case all the bulky inorganic precipitates were examined for N. Small quantities were retained, but extraction with HCl and repetition of the silver precipitations gave no further quantities of the diamino-acids.

The weights of basic amino-acid in the proteins calculated from the highest analytical figure gave the following percentage amounts:

	Depocasein	L.W.S. casein	Pure caseinogen [Vickery & White, 1933]
Arginine	3.32	3.15	3.85
Histidine	1.58	1.53	1.83
Lysine	4.49	4.52	6.25

In comparison with the data of Vickery & White [1933] all the data are lower. Those for lysine are undoubtedly too low. The discrepancy for lysine is attributable to the presence of nitrate in the lysine fractions which had usually to be left over for many days before making lysine phosphotungstate. During this time reduction to nitrite probably occurred with consequent destruction of part of the lysine. In the earlier paper by Vickery & Leavenworth the use of silver nitrate was condemned, but in the later paper by Vickery & Block its use was advised.

The molecular ratios of the amino-acids in the proteins obtained by dividing the percentage figures by the mol. wt. are:

	Histidine	Arginine	Lysine
L.W.S. casein	0.0100	0.0181	0.0272
Depocasein	0.0102	0.0191	0.0300

which work out, more particularly for depocasein, as histidine : arginine : lysine = 1 : 2 : 3.

Depocasein and L.W.S. casein from these analyses thus show no important difference in their contents of basic amino-acids. In comparison with Vickery and White's figure for lysine in pure caseinogen depocasein contains a smaller quantity.

Comparison of these data with those by Van Slyke's method by converting the N figures into weights of protein and amino-acids gives:

	L.W.S. casein		Depocasein	
	Vickery method	Van Slyke method	Vickery method	Van Slyke method
Arginine	3.15	5.08	3.32	3.72
Histidine	1.53	1.25	1.58	4.61
Lysine	3.98	8.75	4.38	6.95

There is little correspondence. Plimmer & Lowndes [1938], who made an examination of the basic amino-acids in the Van Slyke solution, were unable to give an explanation of this discrepancy. In the Van Slyke method there is no attempt at purification of the components in either the mono- or di-amino fractions. Arginine phosphotungstate is definitely not completely precipitated and yet the Van Slyke figure is higher. Monoamino-acids may be thrown down by phosphotungstic acid, particularly proline. The combination of these factors accounts for variability in the Van Slyke figures.

#### *Determination of tyrosine and tryptophan*

The method of Folin & Looney [1922] and its modification by Folin & Ciocalteu [1927] have been used for the determination of tyrosine and tryptophan in both L.W.S. casein and depocasein.

(a) *Folin & Looney method.* The results by this method for L.W.S. casein were very variable. Tyrosine values ranged from 1.35 to 3.76 %, and tryptophan values from 1.20 to 1.51 %.



From the consideration that the protein contains three times as much tyrosine as tryptophan, estimations of each amino-acid were made separately. For tyrosine, 2 or 3 ml. of the hydrolysate were precipitated with 2 ml. reagent. Colours comparable with standards of 1 mg. of tyrosine were secured and the results were concordant. For tryptophan, 6 or 8 ml. of hydrolysate were necessary. Complete precipitation of tryptophan mercuric sulphate was not effected with 2 ml. of reagent. Satisfactory results were obtained with 3 ml. reagent, and were not improved with 4 ml. Mercuric sulphate appears to combine with other amino-acids. 2 ml. reagent contain 200 mg.  $\text{HgSO}_4$ , a large excess over the amount required to throw down the tryptophan present. This inference is borne out on addition of the  $\text{Na}_2\text{CO}_3$  solution; with the tyrosine standard there is precipitation of  $\text{HgCO}_3$ , with the hydrolysed solution the precipitate was small and sometimes absent. The mercuric amino compounds appear not to be easily decomposed by  $\text{Na}_2\text{CO}_3$ .

The best results by this modification for L.W.S. casein were:

Tyrosine	7.09 and 7.01 %
Tryptophan	1.77 and 1.76 %

(b) *Folin & Ciocalteu method.* The chief modifications of the Folin-Looney method consist in hydrolysis by means of 20 %  $\text{NaOH}$ , the use of increased quantities of  $\text{HgSO}_4$  and phenol reagents, and the estimation of tyrosine by Millon's reagent. By this method the values for L.W.S. casein were tyrosine 6.7 % and tryptophan 0.36 %. The tyrosine value compares favourably with that by baryta hydrolysis, but the tryptophan value is about  $1/5$ . Hydrolysis by 20 %  $\text{NaOH}$  seems to produce extensive decomposition of tryptophan. The stability of tryptophan to  $\text{NaOH}$  hydrolysis was therefore tested. Complete destruction occurred on boiling tryptophan alone, but on boiling with gelatin the destruction amounted to only 35 %.

(c) *Estimation by tryptic hydrolysis.* A solution of L.W.S. casein was submitted to hydrolysis by trypsin in faintly alkaline solution and tryptophan estimated at various times of digestion by the Folin-Ciocalteu procedure. The content of tryptophan was found to be 1.75–1.87 % indicating that baryta hydrolysis gives correct results.

(d) *Combination of the Folin-Looney-Ciocalteu methods.* Determination of tyrosine and tryptophan has been effected by baryta hydrolysis of 1 g. protein, and precipitation of the solution with 4 ml. of  $\text{HgSO}_4$  reagent. Tyrosine was estimated both by the Millon reaction and the phenol reagent. Tryptophan was estimated by the phenol reagent and by bromination as described by Plimmer & Lowndes [1938]. The results calculated as percentage of dry mineral-free protein were:

	Tyrosine		Tryptophan	
	Millon reagent	Phenol reagent	Phenol reagent	Bromination
L.W.S. casein batch 1	—	7.09	1.77	—
	—	7.01	1.76	—
batch 2	5.26	5.19	1.21	1.36
Depocasein from batch 2	8.20	7.08	1.24	1.39
	8.05	7.31	1.23	1.39
	8.21	—	—	—
	8.15	7.20	—	—
Mean		7.68		1.31

The two batches of L.W.S. casein gave different values for tyrosine and tryptophan, indicating a difference between them. This may be explained by its

method of preparation which involves production of lactic acidity by bacterial action. Tyrosine and tryptophan may be split off to a different extent for each batch. Further the commercial preparations may contain varying amounts of fat.

Folin & Ciocalteu give 6.41 % tyrosine and 1.4 % tryptophan in pure caseinogen.

Our figures for tyrosine in depocasein are higher by the Millon method than the phenol method. Preference is given to the phenol method, but the mean figure must be taken. Depocasein differs from caseinogen in containing more tyrosine. Tryptophan is the same in both proteins.

#### *Determination of cystine and methionine*

These amino-acids were estimated for us by Mr J. Lowndes by the latest method of Baernstein [1936] using the alteration in distillation mentioned by Plimmer & Lowndes [1938]. The results compared with caseinogen were:

	Depocasein		Caseinogen
Cystine	0.288		0.36 %
	0.313	0.30 %	
Methionine	2.077		2.89 %
	2.110	2.09 %	

The cystine contents of caseinogen and depocasein are thus the same, but the methionine content of depocasein is 0.8 % lower.

#### *Determination of glycine*

The colorimetric method of Patten [1935] for determination of glycine with *o*-phthalaldehyde was tried with both L.W.S. casein and depocasein. It gave the following results calculated on dry mineral-free protein: L.W.S. casein, 1.0 %; depocasein, 0.2 %. The figure for casein is twice that of Patten. The colour with depocasein was so slight that reproducible readings in the colorimeter were not possible.

#### *The monoamino-acids*

The monoamino-acids in depocasein were investigated by the method of Dakin [1918] using 440 g. = 394 g. of dry ash-free protein. After hydrolysis with 21 % HCl for 19 hr., at which period it was found to be complete, as tested by titration with neutralized formalin, the solution was evaporated *in vacuo* several times to remove excess of HCl and neutralized with NaOH. The precipitate of humin and tyrosine was filtered off and 17.9 g. of recrystallized tyrosine with N = 7.69 % (calc. 7.73 %) were isolated, equivalent to 4.5 % in depocasein. The concentrated solution was then extracted numerous times by simple shaking with an equal volume of butyl alcohol. This procedure was found by Plimmer & Lowndes (unpublished) to be as effective as a continuous extractor with the advantage that the extracted amino-acids are not heated for a long time with butyl alcohol thus preventing the formation of anhydrides. The butyl alcohol was distilled off *in vacuo* after each extraction and the amino-acids separated off. Shaking with butyl alcohol was continued till only very small amounts were dissolved and at this stage the extract was found to contain small quantities of diamino-acids.

The butyl alcohol extract was examined for proline and simple monoamino-acids. The solid amino-acids were boiled with successive portions of absolute alcohol until extraction was complete. The alcohol was evaporated off and the

residue thoroughly extracted with cold absolute alcohol. The residue from this solution was dissolved in hot water and allowed to cool. The precipitate, probably of peptide anhydrides, contained 1.21 g. N and was not further examined. The solution, clarified with charcoal and brought to 500 ml., contained 4.45 g. total N and 2.89 g. non-amino-N. Two methods were used to isolate derivatives of proline from this solution.

(a) *Proline picrate by Town's method* [1928]. 100 ml. were boiled with  $\text{CuCO}_3$ . The dried copper salts were extracted with methyl alcohol and gave 8.8 g. material, from which a solution was obtained containing 0.53 g. of total N and 0.36 g. of non-amino-N. The calculated amount of picric acid was added to 50 ml. of this solution and ultimately 1.4 g. of crystals with m.p. of  $146-148^\circ$ , not altered by recrystallization, resulted. This yield gives 1.14 % of proline in depocasein.

(b) *Proline hydantoin by Dakin's method* [1918]. 100 ml. of the solution were evaporated with 8 g. of  $\text{KCNO}$  (5.2 g. theoretical). The sticky residue after treatment with  $\text{H}_2\text{SO}_4$  and ether finally yielded 1.13 g. of opaque white crystals with m.p.  $156-158^\circ$ . Dakin gave  $165-167^\circ$  as m.p. of *l*-proline hydantoin and  $153-156^\circ$  for the racemic compound. The crystals contained 22.2 % N (calc. 20.0 %). The high figure may be due to the presence of some polymerized cyanic acid. The yield corresponds to 1.18 % in depocasein.

Though only 1.2 % proline was isolated the content in depocasein is undoubtedly greater. The solution from the copper salt after boiling with an equal volume of conc. HCl contained 0.0675 g. non-amino-N which is equivalent to 2.8 %. Caseinogen according to Dakin contains 8 % proline.

The separation of the other monoamino-acids insoluble in absolute alcohol was carried out by the ester method. The ester hydrochlorides dissolved in absolute alcohol were treated with the calculated amount of Na in alcohol and NaCl was precipitated with ether. The solution after being shaken with, and filtered through, dry  $\text{Na}_2\text{SO}_4$  was evaporated *in vacuo*, the alcoholic distillate being collected in dilute HCl to trap low-boiling esters. The esters were then fractionally distilled *in vacuo* using an oil bath and a three-stage mercury diffusion pump, kindly placed at our disposal by Dr Barrow at Birkbeck College. Between the receiver and the pump a trap was inserted immersed in a mixture of solid  $\text{CO}_2$  and alcohol to condense vapours escaping the condenser. The following fractions were obtained:

$M_1$  from alcohol distillate of esters.

$M_2$  at  $58-90^\circ$  oil bath temp.  $60^\circ$  vapour  $T$ . 3.0 mm. Hg pressure.

$M_3$  from trap in freezing mixture.

$M_4$  at  $90-100^\circ$  oil bath  $T$ .  $65^\circ$  vapour  $T$ . 1.5 mm. Hg pressure.

$M_5$  at  $100-120^\circ$  oil bath  $T$ . 0.7 mm. Hg pressure.

No distillate  $120-140^\circ$  oil bath  $T$ .

$M_6$  at  $140-180^\circ$  oil bath  $T$ .

$M_7$  from trap in freezing mixture.

Fraction  $M_1$  was evaporated to dryness and the residue dissolved in water, neutralized and fractionally crystallized. 3.4 g. of crystals with 11.5 % N were obtained and treated by the copper salt method (below). The remainder of the residue was NaCl.

Fractions  $M_2$ ,  $M_3$ ,  $M_4$ ,  $M_5$  were separately boiled with water under a reflux for 15 hr. Except  $M_5$ , which was slightly alkaline and was previously extracted with ether to remove any phenylalanine or serine ester, the solutions were neutral. The amino-acids in these fractions were submitted to extensive frac-

tional crystallization from water and alcohol. Leucine was obtained from each fraction. The final portion had a N content of 10.65 % (calc. 10.69 %). When no further separation of leucine could be obtained, the remainders and fraction  $M_1$  were mixed, dissolved in water and converted into copper salts as described by Brazier [1930]. The copper salts insoluble in cold water gave leucine with 10.64 % N; those soluble in water were separated into copper salts soluble and insoluble in methyl alcohol. Both fractions were found to contain only valine with 11.80 % N (calc. 11.97 %) and leucine with 10.60 % N. The total pure leucine isolated amounted to 38.5 g. The content of leucine in depocasein would thus be 9.8 %. The valine, amounting to 7.9 g., had a rotation of  $[\alpha]_D = 20^\circ$  and would consist of 25 % valine, equal to 0.3 % in depocasein. The remainder corresponds to 1.7 % leucine, making leucine at least 11.5 % of the protein.

Fractions  $M_6$  and  $M_7$  were combined, mixed with water and extracted with ether. The ether-soluble part of  $M_5$  was added. The ether extracts were shaken with conc. HCl and gave on hydrolysis, evaporation and crystallization 2.8 g. of phenylalanine hydrochloride with 17.8 % Cl (calc. 17.6 %). 2.1 g. of phenylalanine were prepared from it by solution in water, neutralization with ammonia and treatment with alcohol. The crystals contained 8.92 % N (calc. 8.48 % N). The high value is probably due to the presence of a little  $\text{NH}_4\text{Cl}$ . The yield is equivalent to 0.6 % in depocasein. The aqueous part was hydrolysed with baryta and examined for dicarboxylic acids and serine. Derivatives of these acids were not isolated, though there was some barium salt insoluble in alcohol and some very soluble amino-acid. Alanine was not found in any of the first fractions of esters. From the large amount of N remaining unextracted by butyl alcohol it is probable that it remained in the aqueous solution.

The residual aqueous solution, after removal of butyl alcohol by evaporation *in vacuo*, was brought to 1 l. and contained 29 g. N. An analysis by Van Slyke's method on 15 ml. without further hydrolysis, gave the following distribution of N:

	% N	Calc. to original depocasein
Amide	4.3	—
Humin	0.7	—
Total N of bases	35.2	19.4
Arginine	14.1	7.8
Histidine	0.3	0.2
Lysine	20.8	11.4
Total monoamino	56.3	31.0
Amino	50.7	28.0
Non-amino	5.6	3.0

The second column, obtained by multiplying the first by 29.0/52.7, shows the distribution as a fraction of the total N of the depocasein taken for hydrolysis. Comparison with the figures for depocasein shows no great difference in arginine; histidine has been reduced and lysine is slightly higher. It is possible that histidine, which is less basic than the two other bases, has been dissolved by butyl alcohol. The monoamino filtrate contains nearly half the total monoamino-acids of the depocasein despite the numerous extractions with butyl alcohol until only very small amounts were removed. Dakin [1920] found that the extraction of the monoamino-acids from gelatin was by no means complete, also observed by Plimmer & Lowndes (unpublished), whether the extraction be made with a continuous extractor or by frequent shaking with fresh portions of butyl alcohol. Dakin's description of the extraction of the amino-acids from hydrolysed caseinogen leads to the conclusion that it is complete. The non-amino-N of the monoamino solution amounting to 3 % on the original depocasein may be due to

the presence of peptide anhydrides or hydroxyproline. Proline was extracted by the butyl alcohol.

The solution should contain only the basic amino-acids and the dicarboxylic acids. It was used only for isolation of the latter. Two methods for the isolation of glutamic and aspartic acids were tried.

(1) *Glutamic acid as hydrochloride*. 500 ml. of the aqueous solution were evaporated and saturated with dry HCl gas. The crystalline precipitate consisted almost entirely of NaCl. It was not possible to obtain the insoluble zinc glutamate. The solution of the precipitate contained only 0.08 g. N.

(2) *Barium aspartate and barium glutamate*. 150 ml. of the solution were treated with cryst.  $\text{Ba}(\text{OH})_2$  and precipitated with alcohol according to Jones & Moeller [1928]. From the insoluble barium salts the insoluble lead salt of aspartic acid was made. The solution of the lead salt, freed from lead, contained N equivalent to 0.14 M aspartic acid. The preparation of the copper salt by adding 1.5 M copper acetate was not successful though the solution contained 0.157 g. N equivalent to 1.4 g. aspartic acid or 2.5 % of the depocasein.

The filtrate from the insoluble lead aspartate would not yield glutamic acid hydrochloride. Hydroxyglutamic acid was also not found. It is not possible to explain the non-isolation of these dicarboxylic acids, especially glutamic acid.

#### *Further examination for dicarboxylic acids*

As these acids could not be isolated from the solution remaining after butyl alcohol extraction a further hydrolysis of depocasein with HCl was carried out with approx. 40 g. material. The hydrolysed solution was evaporated *in vacuo* and found to contain 4.5 g. N = 33.8 g. depocasein.

The basic amino-acids were removed from the solution by precipitation with a slight excess of phosphotungstic acid. The filtrate and washings were evaporated *in vacuo* and the concentrated solution saturated with dry HCl gas. The precipitate, which contained glutamic acid hydrochloride and inorganic matter was first recrystallized as hydrochloride; Cl was removed with  $\text{Ag}_2\text{SO}_4$ ,  $\text{SO}_4$  and  $\text{NH}_4$  ions with  $\text{Ba}(\text{OH})_2$  and after quantitatively removing Ba ions there were isolated 1.2 g. glutamic acid with 9.4 % N (calc. 9.5 % N) which gives a content of 3.6 % in depocasein.

The filtrate from glutamic acid hydrochloride was evaporated *in vacuo* to remove HCl and the residue dissolved in water and treated with ammonium rhodanilate according to Bergmann [1935]. From the rhodanilate 0.4 g. of nearly pure proline with 11.8 % N (calc. 12.2 % N) was isolated which corresponds with 1.2 % in depocasein, comparable with the figure obtained from the butyl alcohol extract. The quantity in depocasein is, however, much larger as the whole of the proline was not crystallized out. The filtrate from proline rhodanilate was treated with pyridine, and the solution precipitated with ammonium reineckate. The reineckate did not give any hydroxyproline in a crystalline state.

Excess reineckate was removed from the solution by means of pyridine, made alkaline with  $\text{NH}_4\text{OH}$  and  $\text{BaCl}_2$  added till there was no further precipitation. The filtrate on evaporation *in vacuo* gave 2.0 and then 0.4 g. impure tyrosine with 7.42 % N. It was then treated with solid  $\text{Ba}(\text{OH})_2$  according to Jones & Moeller [1928] and poured into 5 vol. of 95 % alcohol. The insoluble Ba salts were filtered off, washed, redissolved and reprecipitated with alcohol. From the insoluble Ba salts there were ultimately obtained 3.4 g. copper aspartate, from which 1.0 g. of pure aspartic acid with 10.25 % N (calc. 10.5 % N) equivalent to 3.0 % in depocasein was isolated. Glutamic acid could not be found in the insoluble Ba salts. The alcoholic filtrates from the insoluble Ba salts were

evaporated *in vacuo*, acidified with  $\text{H}_2\text{SO}_4$ , filtered from  $\text{BaSO}_4$ , Cl being removed with  $\text{Ag}_2\text{SO}_4$ , Ag with  $\text{H}_2\text{S}$  and finally  $\text{SO}_4$  quantitatively with  $\text{Ba}(\text{OH})_2$ . On evaporation the solution gave 0.4 g. tyrosine. Together with the previous amounts the total tyrosine obtained by crystallization was 2.8 g. Pure tyrosine weighing 1.8 g. with 7.71 % N (calc. 7.73 % N) was prepared from this by recrystallization. The yield corresponds with 5.4 % in depocasein. Further evaporation gave firstly 2.7 g. impure leucine from which 2.1 g. with 10.6 % N was obtained on recrystallization equivalent to 6.3 % of depocasein, and then fractions with 11.2, 12.0, 11.35 % N and finally a fraction of 0.05 g. with 12.84 % N, which indicated that alanine or other lower amino-acids were present in depocasein. The experiment, essentially for the isolation of the dicarboxylic acids, was also a preliminary trial to isolate amino-acids in sequence from the same hydrolysate.

## SUMMARY

The substance produced from caseinogen by treatment with 1 % NaOH at 37° for 24 hr. and subsequent acidification is not simply a residue of caseinogen from which only phosphorus and amide groups have been removed, but a product containing 55 % of the original N of the protein. The caseinogen is apparently split into two almost equal halves. This half is preferably called depocasein, as it resembles a metaprotein in solubility in 0.1N acid and alkali, but is precipitated by a higher concentration of acid. Its composition in amino-acids is generally very similar to that of caseinogen, but there are a few marked differences:

	Depocasein	Caseinogen
Glycine	0.2	0.5
Alanine	?	1.5
Valine	0.3	7.2
Leucine	11.5	9.4
Phenylalanine	0.6	3.2
Aspartic acid	3.0	4.1
Glutamic acid	3.6	21.0
Proline	1.5	8.0
Tyrosine	7.7	6.4
Tryptophan	1.3	1.4
Cystine	0.3	0.4
Methionine	2.1	2.9
Histidine	1.6	1.8
Arginine	3.3	3.9
Lysine	4.5	6.3

The differences from caseinogen are in tyrosine which is over 1 % higher, methionine which is 0.8 % lower and lysine which is lower; the figure of 4.5 % is, however, too low. Proline is lower, but is probably at least 3 %. There is a difference in aspartic acid but our figure is probably too low. The most striking difference is in glutamic acid. Further analyses of depocasein are contemplated. Depocaseose is under investigation.

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# LXVI. EXPERIMENTS RELATING TO THE CONSTITUTION OF ALLOXAZINE- ADENINE-DINUCLEOTIDE

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It was shown by Das [1936; 1938] that the *d*-amino-acid oxidase of Krebs [1933] was associated with a coenzyme. The latter substance was found by Straub [1938] to contain flavin and later isolated in a pure condition by Warburg & Christian [1938, 1] and reported to be an alloxazine-adenine-dinucleotide.

This coenzyme is becoming increasingly important. Warburg & Christian stated that in addition to its combination with the apoenzyme of *d*-amino-acid oxidase it formed, with other specific proteins, xanthine oxidase and enzymes capable of transferring hydrogen from dihydropyridine nucleotide to oxygen and methylene blue respectively. It is also the prosthetic group of a flavo-protein, isolated from heart muscle by Straub [1939], which was later reported by Straub *et al.* [1939] to be identical with diaphorase (coenzyme factor).

The constitution given to the substance by Warburg & Christian was based on its elementary composition and the liberation of one molecule of adenine by acid hydrolysis and one molecule of lumiflavin by alkaline photolysis. The suggestion was later made that the "old" yellow enzyme was not a natural product but resulted in the course of preparation by the fission of adenylic acid from the dinucleotide [Warburg & Christian, 1938, 2].

The present work was concerned with splitting the substance by mild acid and alkaline hydrolysis and investigating the products formed by enzymic reactions. In connexion with the adenine-containing part of the molecule use was made of the fact that the adenosine-5-phosphoric acids, which are coenzymes of the phosphorylase described by Euler *et al.* [1936, 1, 2], act as activators in the enzymic dephosphorylation of phosphoglyceric acid. The appearance of co-phosphorylase on hydrolysis showed the presence in the molecule of linkages between adenine, carbohydrate and phosphoric acid which are those of muscle adenylic acid. The flavin part of the molecule was investigated, after hydrolysis, in the presence of the protein component of the "old" yellow enzyme, using Warburg's system for the oxidation of Robison ester. In this system a considerable proportion of the original activity of the dinucleotide remained after treatment which completely destroyed its ability to oxidase *d*-alanine. This indicated the production, on hydrolysis, of lactoflavin-5-phosphoric acid and the presence of this residue in the dinucleotide.

## EXPERIMENTAL

### *Alloxazine-adenine-dinucleotide*

The coenzyme was prepared as its Ba salt (24 mg. from 10 kg. yeast) by the method of Warburg & Christian [1938, 1], with whose product it appeared to be identical. The phosphorus content, determined colorimetrically, was 6.5%



( $C_{27}H_{31}O_{15}N_9P_2Ba$  requires P 6.74 %). Spectrophotometric measurements were kindly carried out by Mr G. Günther. At  $\lambda = 3650$  a  $2.8 \times 10^{-5} M$  solution by weight was found to contain  $2.7 \times 10^{-5}$  mols per litre, on the basis of the curve given by Warburg & Christian.

### *Phosphorylase system*

The reaction measured was the dephosphorylation of phosphoglyceric acid. The enzyme preparation was obtained by acetone precipitation from rat muscle extract. Minced rat muscle was extracted twice with twice its weight of water, the extract dialysed 18 hr. against 0.9 % KCl, precipitated with 10 vol. acetone at  $-8^\circ$ , and the precipitate washed with absolute alcohol and dried. For experiment 0.25 g. of the precipitate was extracted with a mixture of 1 ml. 0.04  $M$   $MgSO_4$ , 4 ml. of water and 6 ml. of veronal buffer pH 7.4, and the insoluble material separated by centrifuging.

In each determination were used 0.8 ml. of enzyme solution, 0.5 ml. of phosphoglyceric acid (15 mg. of Ba salt per ml. decomposed with  $M/10$   $Na_2SO_4$ ) and 1.2 ml. of water containing the activator to be investigated. After incubation for 2 hr. at  $30^\circ$  3 ml. of 10 % trichloroacetic acid was added and the protein removed by centrifuging. Free phosphoric acid was determined in 1 ml. of the resulting solution by the method of Teorell [1931].

### *Protein component of the "old" yellow enzyme*

Crude yellow enzyme from yeast was purified by adsorption on alumina  $C-\gamma$  using the method of Weygand & Stocker [1937]. The prosthetic group was then separated by the procedure of Warburg & Christian [1938, 2]. 4 ml. of a solution containing  $0.2 \times 10^{-6}$  mol. of flavin enzyme were mixed with 4 ml. of saturated ammonium sulphate and cooled to  $0^\circ$ . 0.1  $N$  HCl was then added slowly with shaking until the pH of the solution was approximately 2.8. The precipitated protein was washed at  $0^\circ$  at the centrifuge with 7 ml. of half-saturated ammonium sulphate, dissolved at  $0^\circ$  in 1 ml. of  $M/2$  phosphate buffer, pH 7.4 and diluted to 10 ml. The flavin solution was neutralized with  $M/2$   $Na_2HPO_4$  and also diluted to 10 ml. (denoted by F.M. in Table III).

### *Protein component of d-amino-acid oxidase*

From an extract of acetone-dried sheep's kidney [Krebs, 1935] a solid preparation containing the required protein was obtained by the method of Warburg & Christian [1938, 1], separation of the prosthetic group being effected in 35 % saturated ammonium sulphate solution at pH 2.8. 100 ml. of kidney extract yielded 150 mg. of material.

### *Oxidation of Robison ester and d-alanine*

After hydrolysis by  $N/20$  HCl and NaOH the activity of the coenzyme, in combination with the specific proteins, was tested in two different systems, the oxidation of *d*-alanine and of Robison ester.

The experiments were carried out in Warburg vessels at  $30^\circ$ . In each case the gas used was pure  $O_2$  and the centre cups contained 0.2 ml. of 5 % KOH. In the Robison ester system there was used in each vessel  $5.0 \times 10^{-9}$  mol. of the co-enzyme (denoted by F. A. D.) and protein corresponding to that obtained by

splitting the same amount of "old" yellow ferment. For the oxidation of alanine the vessels contained, in the case of hydrolysis with HCl, 10  $\mu$ g. of the coenzyme and 3 mg. of the protein preparation; the coenzyme was in large excess, parallel experiments showing that the rate of O<sub>2</sub> uptake using 2  $\mu$ g. was approximately only 5% less. In the case of hydrolysis with NaOH there was used 0.5  $\mu$ g. of the co-enzyme with 4 mg. of protein.

## RESULTS

### *Production of cophosphorylase from the dinucleotide*

Cophosphorylase experiments were carried out, in the system described, on the intact coenzyme and after hydrolysis of the latter under mild alkaline and acid conditions. Table I summarizes the results obtained.

Table I

Activator	$\mu$ g.-atom P per ml.	% dephosphory- lation
0.0125 mg. adenylic acid	1.66	20.2
0.025       "       "	2.70	32.7
0.050       "       "	3.29	39.9
0.10        "       "	3.95	47.9
0.20        "       "	4.44	53.8
0.025 mg. adenosine triphosphoric acid	2.00	24.2
0.10        "       "	3.13	37.9
0.40        "       "	4.45	53.8
0.636 mg. coenzyme in water	1.84	22.3
0.636 mg. coenzyme after treatment with N/20 NaOH 15 min. at 18° and neutralization with N/20 HCl	3.55	43.1
0.636 mg. coenzyme treated with N/20 NaOH 10 min. at 18° and 5 min. at 95°. Neutralized with N/20 HCl	3.88	47.1
0.636 mg. coenzyme treated with N/20 HCl 10 min. at 95° and neutralized with N/20 NaOH	3.22	39.0
No activator	1.16	14.1

By comparison of the amounts of dephosphorylation when adenylic acid and the coenzyme were used as activators an estimate can be made of the amount of splitting of the latter, in terms of the possible production of adenosine-5-phosphoric acid, under the conditions employed (Table II).

Table II

Treatment	% of theoretical splitting
Coenzyme in water	<5
Coenzyme in N/20 NaOH 15 min. at 18°	28
Coenzyme in N/20 NaOH 10 min. at 18° and 5 min. at 95°	40
Coenzyme in N/20 HCl 10 min. at 95°	20

### *Effect of hydrolysis of the coenzyme on its activity in combination with the protein components of d-amino-acid oxidase and the "old" yellow enzyme*

(1) *Hydrolysis with acid.* The results are shown in Table III. In exps. (a) and (b) different samples of Robison ester and "Zwischenferment" were used in the one system and a fresh protein preparation in the other.

In the oxidation of Robison ester the activity of the coenzyme, after treatment with  $N/20$  HCl, was very similar whether the time of hydrolysis was 30 or 120 min., the rate of  $O_2$  uptake, after subtraction of the blank values, being about 70 % of that with the unchanged substance. Under the same conditions the coenzyme activity with respect to the oxidation of *d*-alanine was completely destroyed.

Table III

(1) Oxidation of Robison ester					
Protein solution	0.25 ml.				
$M/2$ phosphate pH 7.4	0.2 ml.				
Zwischenferment	0.5 ml.	→	→	→	→
Robison ester (0.1 m.)	0.4 ml.				
$N/20$ KCN	0.2 ml.				
$H_2O$	0.45 ml.				0.2 ml.
Coenzyme	—	0.45 ml. F.A.D.	0.45 ml. F.A.D. (30 min. in $N/20$ HCl at 95°)	0.45 ml. F.A.D. (120 min. in $N/20$ HCl at 95°)	0.25 ml. "split" F.M.
$Co_{II}$ (in side cup)	0.2 ml. (0.05 mg.)	→	→	→	→
Time (min.)	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$
(a) 10	8.8	41.9	32.3	32.8	37.6
20	14.0	67.6	51.0	54.1	61.6
30	21.1	91.8	70.5	75.4	83.8
40	25.5	114.3	88.4	94.3	102.6
(b) 10	7.5	45.9	34.0	30.6	—
(2) Oxidation of <i>dl</i> -alanine					
Protein solution	3 mg. in 1 ml. $N/10$ pyrophos- phate pH 8.3	→	→	→	→
$H_2O$	/	/	/	/	1.0 ml.
Coenzyme	1.0 ml. F.A.D.	1.0 ml. F.A.D. (30 min. in $N/20$ HCl at 95°)	1.0 ml. F.A.D. (120 min. in $N/20$ HCl at 95°)	/	/
4.5 % alanine (in side cup)	0.2 ml.	→	→	→	→
Time (min.)	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$
(a) 10	11.3	0	0	0	0
20	24.1	0	0	0	0
30	35.4	0	0	0	0
40	46.7	0	0	0	0
(b) 10	14.2	0	0	0	0

(2) *Hydrolysis with alkali*. Treatment of the coenzyme with  $N/20$  NaOH was carried out at room temperature, as heating at 95° brought about rapid disappearance of the flavine colour. But even under these conditions it did not appear possible to destroy selectively, as in the case of acid hydrolysis, the activity with respect to the oxidation of *d*-alanine. Table IV gives the values for the  $O_2$  uptake. Under the conditions employed, hydrolysis for 15 and 60 min. reduced the rate of  $O_2$  uptake in the Robison ester system to about 75 % and 45 % respectively of that obtained using the untreated coenzyme. In the oxidation of alanine the corresponding values were about 93 and 58 %.

Table IV

(1) Oxidation of Robison ester				
Protein solution	0.25 ml.			
<i>M</i> /2 phosphate pH 7.4	0.2 ml.			
Zwischenferment	0.5 ml.	→	→	→
Robison ester (0.1 <i>M</i> )	0.4 ml.			
<i>N</i> /20 KCN	0.2 ml.			
H <sub>2</sub> O	0.45 ml.			
Coenzyme	/	0.45 ml. F.A.D.	0.45 ml. F.A.D. (15 min. in <i>N</i> /20 NaOH at 18°)	0.45 ml. F.A.D. (60 min. in <i>N</i> /20 NaOH at 18°)
Co <sub>II</sub> (in side cup)	0.2 ml. (0.05 mg.)	→	→	→
Time (min.)	μl. O <sub>2</sub>	μl. O <sub>2</sub>	μl. O <sub>2</sub>	μl. O <sub>2</sub>
10	10.6	45.3	37.8	26.2
20	16.7	71.8	59.6	41.0
30	21.2	94.0	78.9	54.1
40	23.8	112.9	91.8	64.0
(2) Oxidation of <i>dl</i> -alanine				
Protein solution	4 mg. in 1 ml. <i>N</i> /10 pyrophos- phate pH 8.3	→	→	→
H <sub>2</sub> O	/	/	/	1.0 ml.
Co-enzyme	1.0 ml. F.A.D.	1.0 ml. F.A.D. (15 min. in <i>N</i> /20 NaOH at 18°)	1.0 ml. F.A.D. (60 min. in <i>N</i> /20 NaOH at 18°)	
4.5% alanine (in side cup)	0.2 ml.	→	→	→
Time (min.)	μl. O <sub>2</sub>	μl. O <sub>2</sub>	μl. O <sub>2</sub>	μl. O <sub>2</sub>
10	11.7	10.5	6.3	0
20	22.5	20.9	12.7	0
30	33.4	32.2	19.9	0
40	44.3	41.9	27.1	0

After treatment of the coenzyme with *N*/20 HCl at 95 for 30 min. or with *N*/20 NaOH at 18° for 60 min. no visible yellow colour was extracted from the resulting solutions, at pH 5, by benzyl alcohol. As this solvent has been shown by Emmerie [1938] to extract flavin, though not flavin phosphoric acid, it appeared that significant amounts of the former substance were not produced under these conditions.

### CONCLUSIONS

The experiments on the production of cophosphorylase show that adenosine-5-monophosphoric acid is a constituent of the new dinucleotide and is involved with a linkage which is easily hydrolysed, especially under alkaline conditions. The very small amount of cophosphorylase present after using the untreated substance is possibly due to enzymic splitting in the system employed.

The formation of adenosine-5-phosphoric acid, in conjunction with the results obtained in the Robison ester and alanine systems, indicates that in the first stage of acid hydrolysis a considerable proportion of the coenzyme breaks down into two mononucleotides, the reaction which was suggested by Warburg & Christian [1938, 2] to occur enzymically in Lebedew-Saft. The fact that little difference was found in the activity of the coenzyme, with respect to the oxidation of Robison ester, whether treated with *N*/20 HCl for 30 or 120 min. is explained by the slow rate of hydrolysis of lactoflavin-5'-monophosphoric

acid in acid solution [Kuhn & Rudy, 1936]. The difficulty of destroying selectively, in NaOH solution, the activity of the coenzyme as the prosthetic group of *d*-alanine oxidase, may be assigned to the lability of lactoflavin towards alkali.

#### SUMMARY

1. The coenzyme of *d*-amino-acid oxidase has been prepared by the method of Warburg & Christian with whose product it was identical in phosphorus and flavin contents.

2. Hydrolysis of the coenzyme with dilute acid and alkali lead to the production of cophosphorylase, showing the presence in the molecule of adenosine-5-monophosphoric acid.

3. By acid hydrolysis it was possible to destroy the activity of the coenzyme with regard to the oxidation of *d*-alanine while maintaining, to a considerable extent, its ability to transfer hydrogen from dihydropyridine nucleotide. This indicated the liberation, by hydrolysis, of lactoflavin-5-phosphoric acid.

4. It has been suggested that the coenzyme can be split enzymically into two mononucleotides, adenylic acid and lactoflavin-5-phosphoric acid [Warburg & Christian, 1938, 2]. It now appears that this reaction can occur on acid hydrolysis.

I wish to thank Prof. H. von Euler for the facilities he has provided and his interest in this work, and Dr F. Schlenk for advice and criticism.

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# LXVII. COENZYME-LINKED REACTIONS INVOLVING *l*(+)-GLUTAMIC DEHYDROGENASE

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*l*(+)-GLUTAMATE in the presence of its dehydrogenase reduces coenzyme I [Dewan, 1938; Euler *et al.* 1938]. Reduced coenzyme in turn is oxidized enzymically by substrates such as aldehyde, acetoacetate, pyruvate and oxaloacetate [Green & Dewan, 1937; Euler *et al.* 1936: 1937]. The possibility of linkages between glutamate and these systems via coenzyme I would therefore seem likely. Adler *et al.* [1937] have linked the glutamate and alcohol dehydrogenase systems, and Dewan [1938] the glutamate and  $\beta$ -hydroxybutyrate systems.

This present communication describes coenzyme-linked reactions between the *l*(+)-glutamic dehydrogenase system on the one hand and the malic and lactic systems on the other.

## I. PREPARATION OF THE DEHYDROGENASES

A very active preparation of glutamic, malic and lactic dehydrogenases may be obtained from pig heart muscle in the following manner. Two pig hearts are divested of fat and connective tissue, minced in a Latapie, washed 5 times with tap water and ground in a mortar with sand and 250 ml. *M* 0.25 phosphate buffer pH 7.2 for 1½ hr. The thick homogeneous paste is mixed with 350 ml. of the same buffer and centrifuged 5 min. to remove sand and cellular debris. The supernatant is brought to pH 4.6 (slightly yellow to chlorophenol red) with 10% acetic acid and centrifuged for 10 min. The precipitate is rubbed up with distilled water and again centrifuged 10 min. It is finally suspended in 70 ml. *M* 0.5 phosphate buffer pH 7.2. The preparation keeps its activity for about 5 days.

## II. METHODS OF ESTIMATION

Since oxidation of *l*(+)-glutamate results in the production of NH<sub>3</sub> and  $\alpha$ -ketoglutarate [Euler *et al.* 1938; Dewan, 1938] the oxidation can be followed by estimation of NH<sub>3</sub> (Parnas method).

Lactic and malic acids were estimated by the manometric method described by Dewan & Green [1937].

## III. REACTION BETWEEN GLUTAMATE AND PYRUVATE

The reactions were carried out anaerobically in Thunberg tubes at 38° for 1½ hr. Table I shows that glutamate is oxidized by pyruvate only in the presence of the enzymes and coenzyme. Lactic acid was also found only when the whole system was present. Table II shows that the lactic acid found was in close agreement with the theoretical amount calculated on the assumption that 1 mol. of lactic is formed for each mol. of NH<sub>3</sub> produced.

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Table I. *Controls for glutamate-pyruvate oxidoreduction*

Heart enzyme (ml.)	2.0	2.0	2.0	2.0	—
0.3% coenzyme I (ml.)	1.0	1.0	1.0	—	1.0
M/3 l(+)glutamate (ml.)	0.5	0.5	—	0.5	0.5
M pyruvate (ml.)	0.3	—	0.3	0.3	0.3
Water (ml.)	—	0.3	0.5	1.0	2.0
NH <sub>3</sub> (mg.)	0.15	0.01	0	0	0

Table II. *Ratio of observed to theoretical lactic acid calculated from NH<sub>3</sub> produced*

	Lactic acid in $\mu$ l. O <sub>2</sub>	Theory	Ratio
(1)	60	66	0.96
(2)	82	78	1.1
(3)	98	106	0.93

Table III. *Controls for the glutamate-oxaloacetate oxidoreduction*

Heart enzyme (ml.)	2.0	2.0	2.0	2.0	—
0.3% coenzyme I (ml.)	1.0	1.0	1.0	—	1.0
M/3 l(+)glutamate (ml.)	0.5	0.5	—	0.5	0.5
M/3 oxaloacetate (ml.)	0.3	—	0.3	0.3	0.3
Water (ml.)	—	0.3	0.5	1.0	2.0
NH <sub>3</sub> (mg.)	0.18	0.02	0	0	0

Table IV. *Ratio of observed to theoretical malic acid*

	Malic acid in $\mu$ l. O <sub>2</sub>	Theory	Ratio
(1)	122	114	1.1
(2)	86	72	1.1
(3)	88	102	0.9

## IV. REACTION BETWEEN GLUTAMATE AND OXALOACETATE

Oxaloacetate produces similar results when used as oxidizing agent (cf. Table III). Malic acid was also found only when the complete system was present. Table IV shows that the malic acid produced was approximately equal to the theoretical amount calculated on the assumption that 1 mol. is produced for each mol. of NH<sub>3</sub>.

Oxaloacetic acid tends to decompose at 38° to pyruvic acid and CO<sub>2</sub>. There was therefore the possibility of pyruvic acid acting as oxidizing agent. This was not so, however, since lactic acid was not formed under the conditions of the experiment.

## SUMMARY

The anaerobic oxidation of l(+)glutamate by pyruvate and oxaloacetate via coenzyme I has been described.

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# LXVIII. MICRODETERMINATION OF GLUTAMIC ACID

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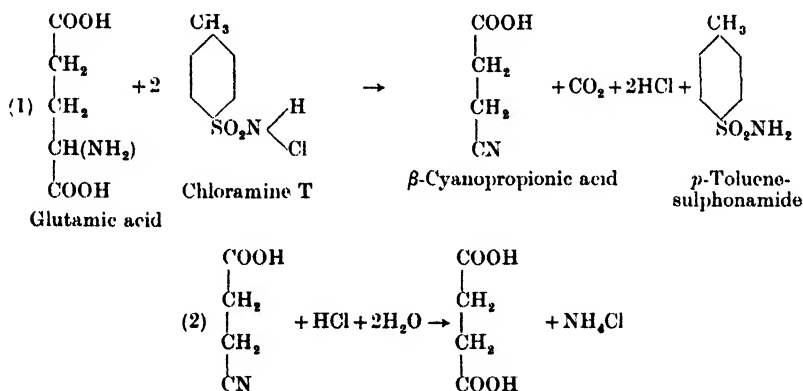
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In the course of experiments on the intermediary metabolism of amino-acids it was found necessary to determine small quantities of glutamic acid. Methods used by previous workers either require large quantities or are unspecific [Foreman, 1914; Jones & Moeller, 1928; Braunstein & Kritzmman, 1937]; on the suggestion of Dr H. A. Krebs a new method has therefore been worked out which is more specific than the older methods and permits the accurate determination of glutamic acid in amounts from 0.1 mg. upwards.

## *Principle of the method*

Glutamic acid is converted into succinic acid by treatment with an excess of chloramine T and subsequent acid hydrolysis [Dakin, 1917]:



The succinic acid is determined manometrically [Szent-Györgyi & Göszy, 1935; Krebs, 1937].

## *Optimum conditions for reaction (2)*

$\beta$ -Cyanopropionic acid was prepared from glutamic acid according to Dakin [1917], and the optimum conditions for its hydrolysis determined by studying the rate of succinic acid formation.

Reaction (2) occurs readily in the presence of strong HCl at 100°. The influence of HCl concentration on the reaction is given in Table I. Hydrolysis is complete within 15 min. when the concentration is at least 12.5%. The effect of the time of heating on the rate of hydrolysis is given in Table II. Since prolonged heating is to be avoided (see discussion of specificity) heating for 15 min. with 12.5% HCl was adopted as the standard procedure.

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Table I. *Influence of HCl concentration on hydrolysis of  $\beta$ -cyanopropionic acid*

Heating time 15 min., 100°. After hydrolysis succinic acid was extracted and determined manometrically.

	mg. $\beta$ -Cyano- propionic acid	% HCl (final conc.)	Succinic acid found ( $\mu$ l. O <sub>2</sub> )	Succinic acid calc. ( $\mu$ l. O <sub>2</sub> )	Hydrolysis %
1	3.22	2.6	153	366	42
2	3.22	6.5	249	366	68
3	3.22	10.4	344	366	94
4	3.22	12.5	363	366	99
5	3.64	15.0	414	414	100

Table II. *Effect of time of heating on rate of hydrolysis of  $\beta$ -cyano-  
propionic acid*

12.5% HCl. After heating succinic acid was extracted and determined manometrically.

	mg. $\beta$ -Cyano- propionic acid	Time of heating min.	Succinic acid found ( $\mu$ l. O <sub>2</sub> )	Succinic acid calc. ( $\mu$ l. O <sub>2</sub> )	Hydrolysis %
1	3.69	0	12.3	418.5	3
2	3.69	2.5	105.3	418.5	25
3	3.69	5	276.3	418.5	66
4	3.69	7.5	345.3	418.5	83
5	3.64	10	393.6	414	95
6	3.64	15	413.1	414	100
7	3.64	20	414.6	414	100

*Optimum conditions for reaction (1)*

Reaction (1) was studied by determining the formation of  $\beta$ -cyanopropionic acid by measuring the succinic acid formed from it on acid hydrolysis.

Dakin [1917] found that reaction (1) took place only when the molar concentration of chloramine T was at least twice that of glutamic acid. We find that an excess of chloramine T does not interfere. The investigation of the influence of time, temperature and pH on reaction (1) showed that quantitative yields of  $\beta$ -cyanopropionic acid were obtained when the reaction was carried out at 40° for 10 min. at pH 4.7. At pH 2.7 the average recovery was 83%, while at pH 7 the recoveries were as low as 38%. Thus it is important to keep the pH in the range of 4-5. At high temperatures (100°) the recoveries average 20%.

The rate of reaction (1) may be followed manometrically by measuring the CO<sub>2</sub> produced. From Table III it can be seen that the theoretical value for CO<sub>2</sub> is obtained within 5 min. It should be noted however that the final CO<sub>2</sub> value is slightly higher than the theoretical. This extra CO<sub>2</sub>, which amounts to 1-2.5%,

Table III. *Rate of CO<sub>2</sub> production from glutamic and  $\beta$ -cyanopropionic acids  
measured manometrically*

Each cup contains 2 ml. 10% chloramine T, 1.1 ml. citrate buffer, pH 4.7, 2 ml. substrate solution. Temp. 40°. 1.51 mg. glutamic acid is expected to yield 230  $\mu$ l. CO<sub>2</sub> according to (1).

Substrate	Time min.	$\mu$ l. CO <sub>2</sub> formed	Yield of CO <sub>2</sub> % of theory
Glutamic acid (1.51 mg.)	5	231.4	100.5
	10	233.1	101.2
	15	235.1	102
	20	235	102
$\beta$ -Cyanopropionic acid (1.62 mg.)	5	1.9	—
	10	1.7	—
	15	1.7	—
	20	2.4	—

implies that glutamic acid is oxidized by chloramine T at some other point in the molecule besides the amino group. Since this "side reaction" occurs simultaneously with reaction (1) it was not found possible to prevent it. As a result of this the corresponding yields of  $\beta$ -cyanopropionic acid are low by 1–2.5%. The extra  $\text{CO}_2$  is not due to further oxidation of  $\beta$ -cyanopropionic acid (Table III).

#### Reagents

(1) Citrate buffer (pH 4.7): 17.65 g.  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ,  $2\text{H}_2\text{O}$  and 8.40 g.  $\text{C}_6\text{H}_8\text{O}_7$ ,  $1\text{H}_2\text{O}$  are dissolved in water and diluted to 50 ml.

(2) 0.1 *M* phosphate buffer (pH 7.4): 17.8 g.  $\text{Na}_2\text{HPO}_4$ ,  $2\text{H}_2\text{O}$  are dissolved in about 500 ml. water and 20 ml. *N* HCl added. The solution is then diluted to 1 l.

(3) 10% chloramine T solution (to be freshly prepared before use).

(4) Conc. HCl.

(5) 5%  $\text{NH}_4\text{Cl}$  solution.

(6) Sat. NaOH solution (50–60%).

(7) Succinoxidase preparation.

The preparation of this enzyme system has been previously described by Krebs [1937]. As noted by Krebs, the enzyme preparation acquires an increasing blank  $\text{O}_2$  uptake on storage. To avoid this, the following procedure may be adopted.

The finely minced pigeon muscle (about 50 g.) is washed with 500 ml. distilled water and filtered by suction through muslin. This procedure is twice repeated. The muscle pulp is sucked as dry as possible after the third washing. For use, a portion of the pulp is suspended in four to five times its weight of 0.1 *M* phosphate buffer, pH 7.4. For storage, it is moistened with distilled water and kept in the refrigerator in a tightly glass-stoppered bottle. Such a preparation remains active without increasing its blank  $\text{O}_2$  uptake for as long as 7–10 days.

The fresh enzyme, prepared by suspension in 0.1 *M* phosphate buffer, usually has a small blank of 1–2  $\mu\text{l}$ .  $\text{O}_2$  per ml. suspension. If such a suspension is allowed to stand in the refrigerator for 4–5 days the blank may increase to as much as 10–15  $\mu\text{l}$ .  $\text{O}_2$  per ml. However, when the enzyme is stored as described above and freshly suspended in 0.1 *M* phosphate buffer as needed the blank is rarely greater than 2–3  $\mu\text{l}$ .  $\text{O}_2$  per ml. after as long as 1 week.

#### Procedure

(1) *Deproteinization*. When tissue slices are employed, it is not necessary to deproteinize, since the small amount of protein present does not interfere. Suspensions of minced tissue are deproteinized by the addition of  $\frac{2}{3}$  vol. of  $\frac{3}{4}N$   $\text{H}_2\text{SO}_4$  plus  $\frac{1}{3}$  vol. of 10%  $\text{Na}_2\text{WO}_4$ . An aliquot of the filtrate is used for the determination.

(2) *Oxidation by chloramine T*. The solution to be analysed is brought to pH 4.7 by the addition of 1–1.5 ml. citrate buffer. 2 ml. 10% chloramine T are added and the solutions well mixed by shaking. They are then placed in a rack and shaken at 40° for 10 min. The reaction is carried out in small Erlenmeyer flasks, or, where tissue slices are employed, the reaction may be carried out in the manometric flask after the removal of the slices and the alkali in the centre well. After 10 min. shaking the containers are removed and placed in an ice bath for 15–20 min. to precipitate most of the *p*-toluenesulphonamide formed as a reaction product, and most of the unused chloramine T. The solutions are filtered, the precipitate is washed with several small volumes of water and the combined filtrate and washings are collected in large test tubes (25 × 200 mm.).

(2A). *Extraction of  $\beta$ -cyanopropionic acid.* In the determination of glutamic acid in blood, tissue extracts and other fluids containing glutathione, it is necessary to separate the  $\beta$ -cyanopropionic acid from another compound derived from glutathione which forms succinic acid on hydrolysis (see discussion on specificity). This is accomplished by extraction with ethyl ether in which the  $\beta$ -cyanopropionic acid is readily soluble, while the glutathione derivative remains in the aqueous layer.

The filtrate described above is acidified with 4 ml. 10 %  $\text{H}_2\text{SO}_4$  and extracted in a continuous extractor of the Kutscher-Steudel [1903] type. With the extractors and conditions used in this study extraction for 1–2 hr. was usually sufficient. 2.5 ml. phosphate buffer are added to the extraction flask and the ether is slowly distilled off. The remaining aqueous solution is then chilled to precipitate the *p*-toluenesulphonamide, which is filtered off, and the filtrate containing the  $\beta$ -cyanopropionic acid is collected in test tubes.

(3) *Hydrolysis of  $\beta$ -cyanopropionic acid.* Conc. HCl is added to the filtrate to make a final concentration of not less than 12.5 %. The tubes are placed in a boiling water bath for 15 min. At the end of this time the tubes are removed and allowed to cool. Conc. NaOH (50–60 %) is added dropwise until the solution becomes hot. At this point 0.5 ml. 5 %  $\text{NH}_4\text{Cl}$  is added and the contents are well mixed. The  $\text{NH}_4\text{Cl}$  decomposes traces of chloramine T, which if present will decolorize the indicator. A few drops of phenol red are now added and the solution is made alkaline to a purple colour. A large excess of alkali should be avoided as the *p*-toluenesulphonamide forms a salt in strongly alkaline solution. The solution is then cooled and transferred to a Kutscher-Steudel extractor.

(4) *Extraction with ether.* The ether must be free from peroxides, otherwise the manometric readings will be inaccurate owing to the decomposition of the peroxides by the catalase in the enzyme preparation [Krebs, 1937]. The alkaline solution is extracted with freshly distilled ether in a Kutscher-Steudel extractor for a time sufficient to remove the remaining traces of *p*-toluenesulphonamide (usually 1–2 hr.). While *p*-toluenesulphonamide in a concentration as high as 0.10 *M* has no inhibiting effect on succinic acid oxidation, it will separate during concentration of the extract if it is not removed and thus make the quantitative transfer of the concentrated solution difficult.

After the period of alkaline extraction, the extraction flask is removed and the contents of the extractor are acidified strongly to phenol red (light yellow-pink colour) with 3 ml. 10 %  $\text{H}_2\text{SO}_4$ . A clean extraction flask with fresh ether is attached and the extraction continued for 2 or more hours, during which the phenol red is also extracted from the aqueous solution.

(5) *Preparation of solution for succinic acid determination.* When the extraction of the succinic acid is complete, 2–3 ml. *M*/10 phosphate buffer are added to the ether solution followed by dropwise addition of 2 *N* NaOH until the aqueous solution assumes the colour of neutral or slightly alkaline phenol red. The ether is distilled off and after the remaining neutral or slightly alkaline aqueous solution has been concentrated further on a steam bath to about 1 ml. it is then transferred to a small graduated cylinder by means of a small pipette. The extraction flask is rinsed with 0.5–2 ml. *M*/10 phosphate and the washings are combined with the solution.

For further details of the succinic acid determination see Krebs [1937].

#### Calculation

The  $\mu\text{l. O}_2$  uptake observed is converted into mg. glutamic acid as follows:

$$\frac{\mu\text{l. O}_2}{112} \times 1.47 = \text{mg. glutamic acid.}$$

*Recovery of known amounts of glutamic acid*

The recoveries of known amounts of glutamic acid from pure solutions are recorded in Table IV. The average recovery is 96 %. The deficit of 4 % is partly due to an over-oxidation of the glutamic acid (as evidenced by the excess  $\text{CO}_2$  production) and partly to unavoidable loss during the various manipulations.

Table IV. *Recovery of glutamic acid*

mg. glutamic acid	% recovery	mg. glutamic acid	% recovery
0.1	99	3.02	91.5
0.36	99	3.60	97.0
0.65	100	5.27	94.4
1.05	95	6.03	98.2
1.32	96.6	8.04	95.2
2.63	92.8	10.05	91.5

The recovery values for glutamic acid added to protein solutions are of the same order as those from pure solutions. Thus, of 8.80 mg. glutamic acid added to a muscle suspension, 8.20 mg. or 94 % were recovered.

The smallest amount of glutamic acid which can be determined is limited by the accuracy of the manometric succinic acid determination. Since 1 mg. glutamic acid is equivalent to 76.2  $\mu\text{l. O}_2$ , as little as 0.1 mg. can be determined by this method. Since the error in the manometric determination is greater with small readings, the method is more accurate with amounts above 1 mg.

*Specificity of the method and interfering substances*

Succinic acid and substances other than glutamic acid which form succinic acid during the course of the procedure will interfere with the determination unless taken into account. Among the substances tested which are likely to occur in biological material, glutamine and glutathione are the only compounds which have been found to yield significant amounts of succinic acid (see Table V).

Table V. *Succinic acid formation from compounds other than glutamic acid*

Compound	Amount (mg.)	Yield of succinic acid %
<i>dl</i> -Proline	29.3	0
$\alpha$ -Ketoglutaric acid	30.9	0
Ornithine-HCl	6.9	0
<i>dl</i> -Alanine	3.3	0
<i>dl</i> -Valine	4.4	0
<i>dl</i> -Leucine	2.5	0
Arginine-HCl	12.5	0
<i>l</i> (-)-Methionine	20.0	0
<i>l</i> (-)-Tyrosine	20.0	0
Aspartic acid	10.0	0
Asparagine	11.4	0
Oxidized glutathione	8.5	17.5
Reduced glutathione	8.5	17.5
Glutamine	1-3.6	70-80

As Weil-Malherbe [1937] has reported that  $\alpha$ -ketoglutaric acid yields succinic acid when autoclaved with  $\text{H}_2\text{SO}_4$ , it should be noted that no succinic acid is formed from relatively large amounts of  $\alpha$ -ketoglutaric acid under the conditions employed in this method.

*Glutathione.* When glutathione is treated with chloramine T under the conditions used for the determination of glutamic acid, it forms a compound or compounds, probably  $\beta$ -cyanopropionylcysteylglycine and the disulphide, which give rise to succinic acid on hydrolysis. In experiments where only negligible amounts of glutathione are present, the glutamic acid can be determined without separating the  $\beta$ -cyanopropionic acid before hydrolysis (step 2A). However, if significant amounts of glutathione are present, as in blood or in certain tissue extracts, it is necessary to remove the  $\beta$ -cyanopropionic acid from the interfering compounds, as described in the procedure (2 A). The recovery of glutamic acid in the presence of glutathione using step 2 A is listed in Table VI.

Table VI. *Recovery of glutamic acid in presence of glutathione*

$\beta$ -Cyanopropionic acid extracted before hydrolysis.

	$\mu\text{l. O}_2$ found	$\mu\text{l. O}_2$ calc.	% recovery
Glutathione (7.2 mg.)	4.9	284*	1.7
Glutamic acid (3.3 mg.)	234	251	94
Glutathione (7.2 mg.) + glutamic acid (3.3 mg.)	242	251	96

\* Calc. for the equiv. quantity of glutamic acid.

*Glutamine.* In quantities of 1 to 3.6 mg. glutamine yields 70–80 % of the theoretical amounts of succinic acid (Table V). The yields were not improved by hydrolysis with acid or alkali under a variety of conditions before the determination. Attempts to convert glutamine into pyrrolidone-carboxylic acid under conditions described by Vickery *et al.* [1935] and subsequent conversion of the pyrrolidone-carboxylic acid into glutamic acid in acid or alkali according to Wilson & Cannan [1937] failed to improve the yields. Since no satisfactory method has been found for converting glutamine quantitatively into glutamic acid, the problem of determining glutamic acid in the presence of glutamine remains unsolved. However, since glutamine occurs only under special conditions [Krebs, 1935], this difficulty will be met with in a few instances only.

*Succinic acid.* When present in appreciable quantities succinic acid can be determined either separately on an aliquot [Krebs, 1937] or it can be removed from the glutamic acid solution by extraction with ethyl ether. Glutamic acid in strongly acid solution is not extracted with ether. Thus a mixture containing 2.62 mg. succinic acid plus 3.96 mg. glutamic acid was acidified with 3 ml. 10 %  $\text{H}_2\text{SO}_4$  and extracted with ether for 30 min. The ether extract was found to contain 2.62 mg. succinic acid, or 100 % recovery, while the aqueous solution when analysed was found to contain 3.78 mg. glutamic acid or 95.5 % recovery.

*Inhibitors of succinoxidase.* Compounds such as malonic, fumaric, oxaloacetic and  $\alpha$ -ketoglutaric [Weil-Malherbe, 1937] acids inhibit the oxidation of succinic acid. The last three compounds only slow up the oxidation, but do not affect the final values. For example, we found that in the presence of 0.125 *M*  $\alpha$ -ketoglutaric acid theoretical results for the added succinic acid were obtained in 60 min.

Neither aspartic acid nor asparagine yields succinic when treated with chloramine T and subsequently hydrolysed (Table VI). However, by analogy with reactions (1) and (2) it might be expected that they would yield malonic acid. To test this, solutions of aspartic acid and asparagine were treated according to the outlined procedure. These were then added to an enzyme preparation and the  $\text{O}_2$  uptake of a known amount of added succinic acid was determined. The theoretical malonic acid concentration in the case of aspartic acid was 0.012 *M*, of asparagine 0.014 *M*. However, the theoretical  $\text{O}_2$  uptake of the added

succinic acid (0.0025 *M*) was observed in the same period of time as in the controls. Thus neither aspartic acid nor asparagine interferes with the determination of glutamic acid.

### *Application*

An example showing the application of the method to studies in intermediary amino-acid metabolism is shown in Table VII in which are listed data from experiments on transamination ("Umaminierung" of Braunstein & Kritzmann [1937]). It is to be seen that the formation of glutamic acid from

Table VII. *Rate of anaerobic glutamic acid formation and disappearance in pigeon breast muscle*

1 part pigeon breast muscle suspended in 5 parts *M*/10 phosphate buffer (*pH* 7.4); final substrate concentration = 0.016 *M*.

Glutamic acid formation from $\alpha$ -keto-glutaric and <i>l</i> (+) alanine		Glutamic acid disappearance	
min.	mg. glutamic acid	min.	mg. glutamic acid
0	0.61	0	8.82
10	6.67	60	4.57
			(pyruvic acid added)
20	5.40	60	8.20
40	5.00		(no pyruvic acid added)

$\alpha$ -ketoglutaric acid and *l*(+)alanine proceeds very rapidly in minced muscle and reaches a peak within the first 10 min.; thereafter the glutamic acid formed slowly disappears. When glutamic acid alone is added to muscle it also disappears slowly, but when pyruvate is added 50% disappears in 60 min. The findings, which are being investigated further, in general confirm those reported by Braunstein & Kritzmann [1937].

The glutamic acid contents of different tissues are listed in Table VIII. The tissues were minced shortly after the death of the animal and extracted with

Table VIII. *Glutamic acid content of tissues*

	mg. glutamic per 100 g. wet wt.
Pigeon breast muscle	34
Pigeon gizzard	94
Guinea-pig kidney	94
Guinea-pig liver	47
Sheep brain (hemispheres)	80
Sheep heart muscle	171
Blood plasma (human)	2.8

2 vol. of boiling water. The glutamic acid contents of these tissues are remarkably high. In contrast, blood plasma has a very low glutamic acid content. Determinations of the glutamic acid content of the erythrocytes from the same specimen of blood show a value of the order of 20 mg. 100 ml.

A sample of human urine was found to contain 4.3 mg. 100 ml. of glutamic acid.

### SUMMARY

A micro method for the determination of glutamic acid in biological material is described. Glutamic acid is oxidized by chloramine T to  $\beta$ -cyanopropionic acid; the latter is hydrolysed to succinic acid; succinic acid is determined

manometrically by means of a succinoxidase preparation. The method is highly specific; no other compound except glutamine occurring in biological material has been found to react.

The glutamic acid content of different tissues has been found to be remarkably high, the highest value observed being 171 mg. per 100 g. heart muscle.

I wish to express my thanks to Dr H. A. Krebs for suggestions and help throughout this study.

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# LXIX. THE ISOLATION OF TWO TRANSFORMATION PRODUCTS OF TESTOSTERONE FROM URINE

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INCREASES in the amount of androgenic material, assayed biologically, which could be extracted from human urine following administration of androgens, have been reported by Buhler [1933], by McCullagh *et al.* [1938], who treated a eunuch with urine extract, and by Kochakian [1937]. These were small and uncertain, and were produced by low doses. Dorfman & Hamilton [1939], using efficient methods of extraction, have recently reported three cases in which large increases in the amount of androgenic material occurred as a result of the administration of testosterone propionate. Prof. J. W. Cook has carried out chemical work on these extracts [Cook *et al.* 1939]. Callow *et al.* [1939, 1, 2] have investigated independently a series of cases in which large doses of hormones were administered. One of these patients, a man under the care of Dr E. P. Sharpey-Schafer, was receiving 100 mg. of testosterone propionate daily. A clinical account of this case has been published elsewhere [Schrire & Sharpey-Schafer, 1939, case 3]. During the administration of testosterone propionate the excretion of 17-ketosteroids (measured colorimetrically by the modified Zimmermann reaction described by Callow *et al.* [1938]), and of androgenic activity, increased to about four times the level of that of a subsequent control period, when this excretion was within normal limits, so that there can be little doubt that the increase was directly caused by the injections; sufficient extract was available for chemical separation of the 17-ketosteroids, with which the urine was enriched, to be attempted.

The neutral fraction of the benzene extract of acid-hydrolysed urine was separated into ketonic and non-ketonic fractions by the Girard-Sandulesco reagent P. The ketonic fraction after chromatographic adsorption followed by elution of the column yielded androsterone and a compound which was identified as aetiocholan-3( $\alpha$ )-ol-17-one (Fieser's nomenclature [1937]; the "epi-oxy-aetiocholanon-17" of Ruzicka & Goldberg [1935]). A total of 60 mg. of crude androsterone and 58 mg. of crude aetiocholan-3( $\alpha$ )-ol-17-one were obtained from 7½ l. of urine or about 6½ days' output.

As a control, the extract from 50 l. of a bulk collection of normal men's urine was put through the same process. A total of 60 mg. of androsterone and 70 mg. of crude aetiocholan-3( $\alpha$ )-ol-17-one were separated. The isolation of the latter compound from normal urine has not previously been reported.

Thus from the patient receiving testosterone propionate 8 mg./l. of crude androsterone and 7.7 mg./l. of crude aetiocholan-3( $\alpha$ )-ol-17-one were isolated, while from the normal men's urine the same two compounds were obtained in yields of 1.2 and 1.4 mg./l. respectively. The androsterone which was isolated accounts for about 74 % of the androgenic activity, assayed on capons, of the extracts of the "testosterone urine", and about 45 % of the activity of the normal urine extract.



## EXPERIMENTAL

All melting points in this paper were observed under the microscope on a slide on an electrically heated stage (Kofler's micro-melting point apparatus). The optical rotations were measured in absolute alcoholic solution in a 4 dm. tube.

*Collection and extraction of urine.* 24-hr. collections of urine were made for 8 days while the patient was receiving 100 mg. of testosterone propionate daily by intramuscular injection, beginning on the second day of injection. Toluene was used as preservative, and the completed collections were stored at 0° until hydrolysis, which was carried out not more than 5 days after collection. Before hydrolysis the pH of all the samples was 6, indicating that little, if any, putrefaction had taken place. The 24-hr. collections were hydrolysed, extracted, and separated into neutral, acidic, and phenolic fractions by the routine method in this laboratory [Callow, 1936]. Colorimetry indicated a content of 17-ketosteroids varying from 28 to 45 mg., average 38 mg. daily. By capon assay (for which I am indebted to Mr C. W. Emmens) the average androgen content was 126 i.u. per day.

The neutral fractions, equivalent to 6½ days' excretion or 7½ l., were combined and taken up in about 15 ml. of methanol. Next day the solution was filtered from traces of insoluble material; evaporation of the filtrate gave 0.54 g. of neutral fraction. Treatment with the Girard-Sandulesco reagent P (carbohydrazidomethylpyridinium chloride) [cf. Callow & Callow, 1938] gave a "ketonic fraction" weighing 0.255 g. and a "non-ketonic fraction" weighing 0.24 g. Colorimetry indicated that the ketonic fraction contained about 65% of 17-ketosteroids.

*Separation by fractional adsorption.* The ketonic resin was dissolved in 10 ml. of carbon tetrachloride (A.R.), left overnight and filtered. The filtrate, diluted to about 25 ml., was put through a 26 × 1.5 cm. column of alumina (Merck's "Aluminium oxide standardized according to Brockmann") in carbon tetrachloride. It was developed in the usual way with about 1.5 l. of carbon tetrachloride, and then with carbon tetrachloride containing successively 0.1, 0.2 and 0.3% of absolute alcohol. Successive portions of 200–500 ml. of the eluate were evaporated. The pure carbon tetrachloride eluate gave only a trace of coloured gummy material. The 0.1% alcohol solution caused a narrow yellow band to move rapidly down the column, and evaporation of 300 ml. of eluate gave 11.7 mg. of gummy residue. Elution with the 0.2 and 0.3% alcohol solutions gave crystalline residues. The weights, volumes of eluate, and the melting points and specific rotations of the residues are shown in Table I. After these fractions had been removed, no more solid was obtained by further elution with 0.3% of alcohol in carbon tetrachloride, and subsequent treatment with 600 ml. of 0.5% and 500 ml. of 1.0% alcohol solutions yielded only 6 mg. of gummy material.

Table I

Frac- tion	Vol. and nature of solvent	Wt. crude crystals mg.	M.P. ° C.	Optical rotation		Main product subsequently identified as
				[ $\alpha$ ] <sub>D</sub>	[ $\alpha$ ] <sub>5461</sub>	
I	350 ml. 0.2% EtOH in CCl <sub>4</sub>	42	175–181	+92°	+104°	Androsterone
II	225 ml. " "	18	170–184 (recryst.)	+85°	+89°	"
III	250 ml. 0.3% EtOH in CCl <sub>4</sub>	21.5	130–146	+89°	+124°	Aetiocholan- 3( $\alpha$ )-ol-17-one
IV	225 ml. " "	15.6	115–151	+91°	+98°	"
V	225 ml. " "	13	136–149	+102°	+122.5°	"
VI	240 ml. " "	5.7	—	—	—	"

*Characterization of androsterone*

The specific rotation, and the characteristic habit of subliming on to the coverslip during the determination of the M.P. on the micro-melting point apparatus suggested that both fractions I and II were androsterone. After two recrystallizations from methanol, fraction I yielded 24 mg. of crystals, M.P. 183–184° (soft at 179°). A mixed melting point with an authentic specimen of androsterone was 181–184.5°. The identity of the compound as androsterone was confirmed by preparation of the oxime. 10 mg. of the ketone, 6 mg. of hydroxylamine hydrochloride and 6 mg. of anhydrous sodium acetate were boiled under reflux in alcoholic solution for 2½ hr. The solvent was removed under reduced pressure, and the crystalline residue taken up in about 3 ml. of acetone, the solution filtered and evaporated to about 0.5 ml. On cooling 8 mg. of the oxime, M.P. 203–206 were obtained. After recrystallizing from acetone it had M.P. 206–212.5° and mixed M.P. with an authentic specimen of androsterone oxime 208–212.5°.

Fraction II after recrystallization from methanol had M.P. 182–184, which was not depressed by admixture with an authentic specimen of androsterone.

*Identification of aetiocholan-3(α)-ol-17-one*

The colour reaction of material from fraction III showed the absorptiometric spectrum characteristic of a 17-ketosteroid [cf. Callow *et al.* 1938]. 21 mg. of the crude fraction III were treated with a hot solution of 100 mg. of digitonin in 10 ml. of 50 % aqueous alcohol. Next day the slight precipitate was removed by centrifuging and the supernatant liquid was diluted with water and extracted with ether; the ether extract, washed with water, and dried over sodium sulphate, yielded on evaporation a residue, 19 mg., which was recrystallized from aqueous methanol. The crystals showed a characteristic transition from a needle-shaped to a platy crystalline form at 137–139°, with partial fusion and resolidification. The final M.P. was 150°. Further recrystallization gave material with M.P. 147–151° and  $[\alpha]_D + 100^\circ$ ;  $[\alpha]_{5461} + 130^\circ$  (EtOH); and finally with M.P. 151–152° (transition point 140–142°). This M.P. was not depressed by admixture with an authentic specimen of aetiocholan-3(α)-ol-17-one, M.P. 152–153° (transition point 140°). Found: (Weiler) C, 78.2; H, 10.0 %; calc. for  $C_{19}H_{30}O_2$ : C, 78.6; H, 10.4 %.

*Preparation of aetiocholan-3(α)-ol-17-one benzoate*

Material, M.P. 147–151°, obtained by evaporation of the mother liquors from the last recrystallization of the above aetiocholan-3(α)-ol-17-one was dissolved in 0.25 ml. of dry pyridine and two drops of benzoyl chloride added. After heating on the water bath for 5 min., the mixture was cooled, and diluted with water drop by drop. The sticky crystals which separated were well washed with water, and recrystallized from aqueous methanol, from which characteristic bundles of needles, M.P. 140–160°, separated. After four recrystallizations from aqueous methanol, it had M.P. 153–161°. There was insufficient for further purification.

For comparison a specimen of aetiocholan-3(α)-ol-17-one benzoate, which has not been described before, was prepared from the authentic hydroxyketone as described above. After recrystallization from aqueous methanol, and from absolute methanol, it had a constant M.P. 161.5–163.5°, and crystallized well in long needles similar to those already described. The melting point of the sample prepared from the urine extract was not depressed by admixture with this authentic specimen.

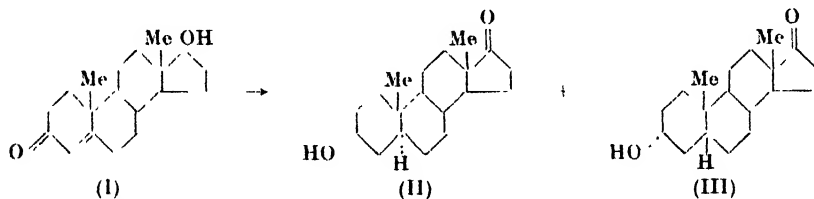
Fractions IV, V and VI were combined, after preliminary measurements of their melting points and specific rotations, and recrystallized from benzene. The top fraction partially melted and recrystallized at  $137^{\circ}$  and finally melted at  $152^{\circ}$ . The last fraction, obtained by evaporation of the mother liquors, m.p.  $142-148^{\circ}$ , yielded a benzoate, m.p.  $151-160^{\circ}$ . There was insufficient for further purification, but it was similar in crystalline form and behaviour to the substance identified above as aetiocholan-3( $\alpha$ )-ol-17-one benzoate. The top and intermediate fractions from the benzene recrystallizations were united (15 mg.) and treated with a hot solution of 80 mg. of digitonin in 10 ml. of hot 50% aqueous alcohol. After standing overnight there was only a slight precipitate, and 11 mg. of material were recovered from the filtrate. This was treated with acetic anhydride in presence of pyridine on the water bath for 30 min. The mixture was cooled, water was added and the oil which separated was extracted with ether; the extract was washed with aqueous  $\text{Na}_2\text{CO}_3$  and with water, dried and evaporated. The residue, twice recrystallized from aqueous methanol, had m.p.  $91-94.5^{\circ}$ . The m.p. of a slightly impure authentic specimen of aetiocholan-3( $\alpha$ )-ol-17-one acetate (m.p.  $89.5-94^{\circ}$ ) was not depressed by admixture with this specimen.

The normal urine extract used as a control was part of a bulk collection of 250 l. of normal men's urine worked up by Messrs Boots Pure Drug Co. This opportunity is taken to acknowledge their generosity in undertaking this work. The urine was collected with Brilliant Green as preservative, hydrolysed with HCl and extracted with chloroform. The chloroform was evaporated, and the extract sent to this laboratory, where it was taken up in benzene, and separated into neutral, acidic and phenolic fractions in the usual way. Colorimetry of the neutral fraction indicated 8 mg./l. of 17-ketosteroids, and capon assay 26 i.u./l. of androgenic activity. The neutral fraction of the extract from 50 l. of urine was separated into "ketonic" and "non-ketonic" fractions. These weighed 0.48 and 1.12 g. respectively. The ketonic fraction was taken up in carbon tetrachloride, and put through a column of alumina. The column was developed with carbon tetrachloride, and carbon tetrachloride containing 0.1, 0.2 and 0.3% of absolute alcohol, as described for the "testosterone urine". Development with any one solvent was continued until evaporation of about 250 ml. of eluate gave a residue weighing less than 10 mg. Evaporation of 310 ml. of the 0.1% alcohol eluate gave 49 mg. of a semicrystalline residue. After treatment with charcoal and repeated recrystallization from aqueous methanol, 6 mg. of a product, m.p.  $142-150^{\circ}$ , were obtained. This was subsequently identified as *transdehydroandrosterone*. 15 mg. of material from the mother liquors were treated with 60 mg. of digitonin in 70% aqueous alcohol. The precipitated digitonide was decomposed in the usual way with pyridine, and yielded 2.5 mg. of a crystalline substance, m.p.  $136-145^{\circ}$ . This was combined with the material obtained by fractional crystallization and converted into the benzoate. This, after recrystallization from ethyl acetate, was recognized as *transdehydroandrosterone benzoate*. It then had m.p.  $242-248^{\circ}$ , not depressed by admixture with an authentic specimen. Elution with a total of 810 ml. of 0.2% alcohol in carbon tetrachloride yielded 60 mg. in all of crystalline residue. This was identified as *androsterone* by its m.p., mixed m.p. and property of subliming. The next fraction, obtained by elution with 370 ml. of 0.3% alcohol in carbon tetrachloride, yielded 70 mg. of crystalline material. After repeated recrystallization from aqueous methanol, the m.p. became constant at  $144-147^{\circ}$ . A mixed m.p. with an authentic specimen of aetiocholan-3( $\alpha$ )-ol-17-one was  $145-150^{\circ}$ . The identity of the compound was confirmed by preparation of the benzoate. This had m.p.  $159-162^{\circ}$ , and a mixed m.p.  $162-163.5^{\circ}$  with an authentic specimen of aetiocholan-3( $\alpha$ )-ol-17-one benzoate.

## DISCUSSION

The isolation of androsterone and the stereoisomeric compound aetiocholan-3( $\alpha$ )-ol-17-one from the urine of a man receiving testosterone propionate, and, in much smaller quantities, from normal men's urine, is particularly interesting from the point of view of determining the parent substances of the steroid compounds excreted in urine, whether the former have their origin in the gonads or the adrenals. Testosterone is the only androgen which has been isolated from gonadal tissue, but there is no evidence that it is ever excreted in urine. This work owed its inception to the hope that some degradation product might be recognized as an index of testosterone production, just as pregnanediol is recognized as an index of progesterone production [cf. Venning & Browne, 1936; 1937; Venning, 1937]. Since androsterone has been isolated from normal women's urine [Callow & Callow, 1938] and aetiocholan-3( $\alpha$ )-ol-17-one from the urine of a woman with adrenal hyperplasia [Butler & Marrian, 1938], the meaning of the excretion of these two compounds must remain a matter of speculation until further work has been done. The possible occurrence of aetiocholan-3( $\alpha$ )-ol-17-one in the urine of normal women is now being investigated.

Hartmann & Locher [1935] suggested that pregnanediol and *allopregnanediol*, which can be isolated from the urine of pregnant women, were derived from progesterone by reduction. A completely analogous process in ring I can be postulated for the degradation of testosterone (I) to androsterone (II) and aetiocholan-3( $\alpha$ )-ol-17-one (III), involving the reduction of the 3-keto-group to give a 3( $\alpha$ )-hydroxy compound, and of the 4:5 double bond to give both the two possible configurations at position 5, with oxidation of the 17-hydroxyl group.



Butler & Marrian [1938] suggested that the aetiocholan-3( $\alpha$ )-ol-17-one which they isolated from the urine of a woman with adrenal hyperplasia was derived by partial oxidation from pregnane-3( $\alpha$ ):17:20-triol, which they isolated from the same urine. The finding of the former compound as a degradation product of testosterone does not, of course, disprove this hypothesis, but it shows that another mechanism is possible.

The degradations of testosterone to androsterone and aetiocholan-3( $\alpha$ )-ol-17-one now experimentally demonstrated may be compared with the speculative scheme of degradation of male hormones in the body recently put forward by Marker [1938]. He suggested that androsterone and aetiocholan-3( $\alpha$ )-ol-17-one were stages in the reduction of  $\Delta^4$ -androstene-3:17-dione. This scheme would be in accordance with the facts, provided that  $\Delta^4$ -androstene-3:17-dione were derived from testosterone, and not the reverse, as assumed by Marker. He also suggested that *transdehydroandrosterone* ( $\Delta^5$ -androstene-3( $\beta$ )-ol-17-one) is derived from  $\Delta^4$ -androstene-3:17-dione. In the case investigated there was actually no large increase in the excretion of *transdehydroandrosterone* during the administration of testosterone propionate. From the behaviour of the extract from normal urine, it is known that this compound is eluted from the adsorption column with carbon tetrachloride containing 0.1% of alcohol. The

corresponding fraction from the "testosterone urine" was a gum which weighed only 11.7 mg., and was obviously a mixture. The yield of this fraction from seven times the volume of normal men's urine was 49 mg. of semicrystalline material, from which 8 mg. of *trans*dehydroandrosterone were isolated. *trans*Dehydroandrosterone is more probably derived from the adrenal cortical secretions [cf. Callow, 1938].

When this work was nearing completion it was learnt from Prof. J. W. Cook that he had isolated androsterone as a degradation product of administered testosterone, and accounts of both his and our investigations were given to the meeting of the Biochemical Society on 10 February 1939 [Cook *et al.* 1939; Callow *et al.* 1939, 1].

#### SUMMARY

Androsterone and aetiocholan-3( $\alpha$ )-ol-17-one have been isolated from the urine of a man receiving 100 mg. of testosterone propionate daily in yields of 8 and 7.7 mg./l., respectively. The same two compounds have been obtained from normal men's urine in yields of 1.2 and 1.4 mg./l.

I have pleasure in thanking Dr E. P. Sharpey-Schafer of the British Post-Graduate Medical School for putting the urine from his case at my disposal, and Dr D. Beall for arranging its collection and transport. I am greatly indebted to Prof. Ruzicka for a specimen of aetiocholan-3( $\alpha$ )-ol-17-one, which he worked up from the semicarbazone so that a comparison with the natural product might be made, and to Prof. G. F. Marrian for a specimen of the acetate. Finally I have to acknowledge much encouragement and helpful criticism from my husband, Dr R. K. Callow.

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# LXX. THE PHOSPHORYLATION OF CARBOHYDRATE IN LIVING CELLS

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It has been shown previously that the fermentation of sugar by living yeast is accompanied by a decrease in the amount of orthophosphate present in the cell as compared with the resting value, and that this decrease persists until the sugar added has been fermented, at which stage the orthophosphate tends to resume its original value [Macfarlane, 1936]. This coincidence of phosphorylation with fermentation provided evidence of what had hitherto been an assumption, namely, that the fermentation of sugar by living yeast takes place in a manner analogous to that found in yeast juice and other cell-free fermentations, through the intermediate formation and decomposition of hexosephosphoric esters. The scale and technique of the previous experiments were not suitable for the identification of any phosphoric esters formed; by working with larger quantities it has now been established that hexosediphosphate is formed during the normal fermentation of sugar by living yeast and that phosphoglyceric acid is also present when the normal fermentation has been inhibited by the addition of NaF. The experiments to be described also illustrate several points of difference between the fermentation of the living cell and that of the dead or cell-free preparation which are interesting in the general question of glycolysis by living cells.

## *Phosphoric esters formed in living yeast during fermentation*

Mild ale yeast was obtained fresh from the brewery vats, washed three times with water and well drained on Büchner funnels. The yeast was used without further pressing, and the water content therefore varied somewhat in different batches. The fermentations were carried out at room temperature in large bottles attached to 500 ml. nitrometers filled with brine. Weighed amounts (100–400 g.) of yeast were mixed with twice their weight of water and the contents of the bottle saturated with CO<sub>2</sub> before connexion to the nitrometers. After an interval of 10 min. to allow equilibrium to be established, the following additions were made to different bottles: (a) water only; (b) NaF, in final concentration 0.05 *M*; (c) sugar, 10 g. per 100 ml. water; (d) sugar, followed by NaF in final concentration 0.05 *M* after the maximum fermentation rate had been established. Enzyme action was stopped by addition of 50 ml. 20% trichloroacetic acid per 100 g. yeast. The precipitate was separated after  $\frac{1}{2}$  hr. by filtration and washed with 5% trichloroacetic acid. From the combined filtrate and washings a sample was removed for P estimations by Briggs's colorimetric method. The total acid-soluble P was determined after ashing, and the content of "pyro" and organic P was calculated from the hydrolysis curve in *N* HCl at 100° according to the equations:

$$\text{"Pyro" P} = \Delta (7 \text{ min.} - 0 \text{ min.}) - \Delta (30 \text{ min.} - 7 \text{ min.}),$$

$$\text{Organic P} = \text{total P} - (\text{ortho P} + \text{"pyro" P}),$$

$$\text{Hexosediphosphate P} = \Delta (180 \text{ min.} - 7 \text{ min.}) \times 1.5.$$

Alkali-labile P was estimated as the increase of ortho-P after 15 min. in *N* NaOH at 22°.

The bulk of the filtrate was treated with an amount of Ba acetate equivalent to the total P present, followed by Ba(OH)<sub>2</sub> to pH 8.0 and one-tenth volume of alcohol. The precipitate was removed by filtration; the filtrate was treated with basic Pb acetate, and the Pb precipitate removed, decomposed with H<sub>2</sub>SO<sub>4</sub> and reconverted into the Ba salt. From P estimations in the successive filtrates the amount of P present in (a) sparingly soluble Ba salts, (b) soluble Ba salts, (c) soluble Ba salts with insoluble Pb salts was calculated by difference.

*Identification of hexosediphosphate.* The sparingly soluble Ba salt consists to a large extent of inorganic (ortho-, meta- and pyro-) P, together with any hexosediphosphate, phosphoglycerate etc. The crude salt was purified by repeated solution in 10 parts H<sub>2</sub>O acidified with HCl to pH 3.6 and reprecipitation, at first as the acid Ba salt by addition of 3 vol. alcohol (the inorganic P tending to remain in solution) and finally by neutralization of the aqueous solution with Ba(OH)<sub>2</sub>. The purification was such as to remove any trace of hexosemonophosphate but owing to the overlapping solubilities and the large excess of pyrophosphate it was exceedingly difficult to obtain a sample of hexosediphosphate free from inorganic P. Proof of the presence of this ester was therefore obtained by the preparation from these fractions of the crystalline phenylhydrazine salt of 6-phosphoglucosazone, which is formed from hexosediphosphate with elimination of one P atom.

*Protocol, Yeast 31. Fermentation of fructose by 650 g. yeast.* The sparingly soluble Ba precipitate, wt. 7.8 g., contained 786 mg. total P, of which 133 mg. were organic. The acid Ba salt was reprecipitated five times with alcohol and finally precipitated by neutralization of the aqueous solution. Wt. 0.81 g. Total P 9.3 %, ortho-P nil. Reducing power (Hagedorn and Jensen) 7.6 %; ketose (Seliwanoff) 5 %.  $[\alpha]_{5461} + 1.8^\circ$ . Ba hexosediphosphate has a reducing power (Hagedorn and Jensen) of 12 % and a Seliwanoff value of 10 %. Calculated from the hydrolysis curve in *N* HCl at 100°, 72 % of the total P content was hexosediphosphate-P.

*Formation of 6-phosphoglucosazone.* 0.2 g. pure Ba hexosediphosphate was converted into the free acid, vol. 5 ml., heated with 0.5 ml. phenylhydrazine and 1.0 ml. glacial acetic acid at 100° for 15 min. and cooled in ice for 20 min. The yield of 6-phosphoglucosazone (phenylhydrazine salt) was 70 % of the theory, with 43 % recovery on recrystallization from hot alcohol and chloroform; m.p. 153–4°. 0.33 g. of the Ba salt described above (31 mg. P), treated similarly, gave 151 mg. osazone, which was recrystallized with 45 % recovery; m.p. 153.6°; P = 5.71 %; required for C<sub>24</sub>H<sub>31</sub>O<sub>7</sub>N<sub>6</sub>P, P 5.68 %. Calculated on the basis of a 70 % yield of phosphosazone, 74 % of the total P in the salt was present as hexosediphosphate, which is in good agreement with the other analytical figures.

*Estimation of hexosediphosphate.* The purification of the hexosediphosphate fraction outlined above entails a considerable loss of organic P and is therefore not suitable for quantitative work, while calculation from the hydrolysis curve of a crude salt containing much labile P involves a considerable error. Attempts to prepare the osazone directly from the crude salt were unsatisfactory. For the routine estimation of hexosediphosphate, therefore, the crude Ba salt was purified once as the acid salt and precipitated as the neutral salt from aqueous solution. The crystalline phospho-osazone was then prepared from an aliquot sample of the salt under standard conditions, and the amount of P as hexosediphosphate was calculated from the weight of osazone obtained on the basis of a 70 % yield.

Table I. *Distribution of acid-soluble phosphorus in yeast*

Exp. no. Additions*	Autofermentation					Sugar fermentation					Sugar fermentation inhibited by NaF									
	29	32	39	39	NaF	29	30	31	32	37	29	39	30	32	29	29	39	30	32	29
Total duration of exp. (min.)	—	—	—	—	—	F	F	F	G	M	F	F	F	G	F	F	F	F	G	F
Duration of exp. after NaF	0	20	20	30	30	25	20	25	20	35	30	30	30	40	30	30	30	40	30	65
CO <sub>2</sub> evolution, ml./5 min./100 g. yeast	—	—	—	10	10	—	—	—	—	—	10	10	10	20	30	45	—	—	—	—
mg. P per 100 g. yeast:	—	—	15	0	0	180	150	135	170	150	<12	<5	<12	0	0	—	—	—	—	—
Total acid-soluble P	178	230	205	202	—	175	164	190	224	153	178	191	174	220	190	—	—	—	—	—
Ortho-P	76	81	80	70	—	35	38	45	43	25	22	37	21	23	7	—	—	—	—	—
"Pyro"-P	63	111	82	73	—	64	—	92	115	68	58	75	—	102	77	—	—	—	—	—
Organic P	39	39	43	60	—	76	73	53	67	60	98	78	98	94	105	—	—	—	—	—
(a) Insoluble Ba ppt.	13	13	15	30	—	37	35	22	28	23	46	31	48	54	35	—	—	—	—	—
Hexosediphosphate P†	—	1	—	—	—	—	—	9.6	7.8	11	—	—	—	3	—	—	—	—	—	—
Phosphoglyceric P‡	0	—	0	0	—	0	0	—	0	—	6	5	7	—	7	—	—	—	—	—
(b) Soluble Ba salts	26	25	28	30	—	39	38	31	29	36	52	47	50	40	57	—	—	—	—	—
P pptd. by basic Pb acetate	—	6	—	—	—	—	12	3	11	—	—	—	—	17	22	—	—	—	—	—
(c) Alkali-labile P	—	0.7	—	—	—	—	—	—	0.9	—	—	—	—	—	1.4	—	—	—	—	—

\* F=fructose; G=glucose; M=mannose.

† Calc. from weight of crystalline osazone obtained from the purified salt on basis of a 70% yield.

‡ Calc. from weight of crystalline BaH phosphoglycerate.



*Identification of phosphoglyceric acid.* Phosphoglyceric acid was isolated quantitatively from the crude sparingly soluble Ba salts as the crystalline acid Ba salt. It was found advantageous to heat the salt first in *N* HCl at 100° for  $\frac{1}{2}$  hr. to decompose labile P compounds. The pH of the solution was then brought to 3.0 with Ba(OH)<sub>2</sub> and 2 vol. alcohol added. The precipitate was dissolved in dilute HCl and 2 vol. alcohol were cautiously added; if any phosphoglyceric acid was present the characteristic crystalline BaH salt was rapidly deposited.

*Other phosphoric esters.* It was anticipated that the fraction with soluble Ba salts would contain some hexosemonophosphate, together with phosphopyruvate etc. Only a small part of the P present, however, was precipitated with basic Pb acetate and this fraction, after reconversion into the Ba salt, contained only 1–3 % P. In spite of repeated attempts at fractionation by Hg acetate, crystallization of brucine salts or alcohol fractionation, the constituents of this mixture could not be separated sufficiently for the identification of any phosphoric ester and no crystalline phenylhydrazine derivative could be obtained.

The values obtained for the distribution of the acid-soluble phosphorus during different types of fermentation are shown in Table I.

*Effect of sugar fermentation on P distribution.* During autofermentation the greater part of the organic P is in the fraction with very soluble Ba and Pb salts, probably of nucleotide nature, but a trace of hexosediphosphate is present. During the fermentation of sugar, whether fructose, glucose or mannose, the organic P is increased in amount, the increase being distributed almost equally between the insoluble and soluble Ba salts. In the insoluble fraction only 50 % of the increase was accounted for by the estimated increase in hexosediphosphate-P (10 mg. P per 100 g. yeast); the method of estimation of this ester however almost certainly gives too low values, since no allowance was made for loss in purification or by the hexosediphosphate carried into the soluble fractions. No phosphoglyceric acid could be isolated either from the resting or fermenting yeast and the amount of alkali-labile P was only slightly raised during fermentation.

*Effect of NaF on distribution of P.* When the fermentation of sugar by living yeast was inhibited by the addition of NaF the presence of phosphoglyceric acid was readily established; the amount found in different batches of the same yeast was constant, c. 7 mg. P per 100 g. yeast, and was not appreciably raised by prolonging the experiment with the NaF-poisoned yeast, e.g. from 10 to 45 min. The ortho-P content however slowly decreased in such yeast (Table I, Yeast 39); this appears to be due to the continued formation of phosphoric esters from glycogen present in the cell, since it was also observed in autofermentation in presence of NaF.

#### *Inhibition of glycolysis with varying concentrations of fluoride*

In the foregoing experiments 0.05 *M* NaF was used, since it was found that with large quantities of yeast this concentration was necessary to produce complete inhibition: even so the inhibition was rarely complete in less than 10 min. The effect of various concentrations of NaF on the fermentation rate is shown in Table II. *M*/200 fluoride caused only 41 % inhibition of glycolysis in the cell, though in yeast juice or muscle extract more than 90 % inhibition is produced by this, or even smaller, concentrations. The sensitivity of glycolysis in different tissues to NaF was shown by Dickens & Šimer [1929] to be extremely variable; in view of the disparity between yeast cells and cell-free extracts the sensitivity may be interpreted as a characteristic of the tissue rather than of the glycolytic enzyme.

Table II. *Percentage inhibition with varying concentrations of fluoride*

2 g. fresh yeast + 9 ml. 10% glucose were fermented 15 min. at 25°; 1 ml. NaF was added and the average fermentation rate measured during the next hour.

NaF molarity	ml. CO <sub>2</sub> /5 min.	% inhibition
0	6.3	—
0.004	4.2	33
0.005	3.7	41
0.006	3.0	52
0.008	1.6	74
0.01	0.6	91
0.02	0	100

*Time relationship between esterification and fermentation in living yeast*

The coincidence of phosphorylation and fermentation has been studied more closely, particularly in the initial and final stages of fermentation of a limited quantity of sugar.

A suspension was made by mixing one part washed fresh yeast with 5 parts water, some of which (A) was used immediately and some (B) after 24 hr. at room temperature. 10 ml. samples of the suspension were placed in fermentation flasks, saturated with CO<sub>2</sub> and connected to nitrometers. After 10 min. incubation, 1 ml. 10% glucose was added and the fermentation rate measured over short intervals. The fermentation was stopped in individual flasks at short intervals by the addition of 3 ml. 20% trichloroacetic acid, and the orthophosphate content of the filtrate estimated. The complete fermentation of 100 mg. glucose would cause the evolution of 25 ml. CO<sub>2</sub>; in the experiment quoted in Table III the rate of fermentation fell, from the maximum of 1.1 ml. CO<sub>2</sub>/2 min.

Table III. *Time relationship between esterification of inorganic P and CO<sub>2</sub> evolution in living yeast*

Yeasts: A, fresh; B, after 24 hr.

Time after addition of glucose	CO <sub>2</sub> rate ml./2 min.		Ortho-P mg./2 g. yeast		Time after addition of glucose min.	CO <sub>2</sub> rate ml./2 min.		Ortho-P mg./2 g. yeast	
	A	B	A	B		A	B	A	B
0	—	—	1.35	1.66	40	0.7	0.9	—	0.92
15 sec.	0	—	1.34	—	46	0.7	0.8	—	0.86
30 "	0	—	1.30	—	48	0.6	0.7	1.01	0.84
1 min.	0	0	1.27	—	50	0.2	0.6	—	0.92
2 "	0.1	0	1.10	—	54	0.2	0.4	—	1.18
3 "	0.2	0	0.93	—	58	0.1	0.2	—	1.12
5 "	0.5	0.3	0.80	—	60	<0.1	0.1	1.09	—
10 "	1.1	0.7	0.75	—	62	—	<0.1	—	1.12
20 "	1.1	1.0	0.83	—	70	—	—	—	1.17
30 "	0.9	0.9	—	0.88					

to the autofermentation rate of less than 0.1 ml. CO<sub>2</sub>/2 min. when approximately 80% of the sugar had been fermented. It will be seen that 3 min. after the addition of glucose 0.42 mg. inorganic P (equivalent to 2.4 mg. glucose as hexosemonophosphate) had disappeared, but that the fermentation did not reach its maximum rate until after the esterification was maximal. The marked lag in the attainment of the maximum rate of CO<sub>2</sub> evolution (Table III) is reminiscent of the Gäranssteig period in fermentation by maceration extracts [Meyerhof, 1918] during which hexosediphosphate is formed. The decrease in orthophosphate in living yeast was maximal in less than 5 min., the level then

remaining steady for  $\frac{1}{2}$  hr., till, with the consumption of the sugar, the  $\text{CO}_2$  rate slackened and finally ceased and the inorganic P rose towards its original value.

The initial value of orthophosphate in yeast B (Table III), i.e. after 24 hr. "resting", was decidedly higher than in the fresh sample; the final value after fermentation was the same in both samples and less than the original value in the fresh yeast. It was found that if a second addition of glucose were made to the yeast, the time taken to attain the maximum rate increased with the interval between the two fermentations; during this interval there was a slow rise in orthophosphate. The point has not been investigated in great detail, but the experiments indicated a "rejuvenating" effect of the fermentation in building up labile P compounds which are slowly hydrolysed in the "resting" cell.

*Independence of orthophosphate content of the cell and that of the medium*

In the experiments so far cited the fermentations were carried out with mixtures of yeast, water and sugar, so that the observed changes in P distribution were internal. The following experiment demonstrates that this change is independent of the concentration of P in the surrounding medium. Weighed amounts (0.5 g.) of yeast were placed in centrifuge pots to which either  $\text{H}_2\text{O}$ , or phosphate buffer pH 6.6, or 1 ml. 10 % glucose or glucose + phosphate was added, the volume in each case being made up to 3 ml. with water. The yeast was well mixed and kept at  $20^\circ$  for 20 min. After centrifuging, the supernatant was separated as quickly and completely as possible with a capillary pipette, and the residue extracted with 5 ml. 0.5 % trichloroacetic acid. Inorganic P was then estimated in the supernatant liquid (external P) and in the trichloroacetic acid extract (internal P). It will be seen (Table IV) that in the presence of glucose,

Table IV. *Internal and external P concentrations in fermenting yeast*

0.5 g. yeast in 3 ml. solution.			
		mg. ortho-P	
Additions		Supernatant liquid	Trichloroacetic acid extract
(1) $\text{H}_2\text{O}$		0	0.46, 0.46
(2) Glucose		0	0.32, 0.32
(3) 0.36 mg. P as phosphate buffer		0.358	0.48
(4) 0.36 mg. P as phosphate buffer + glucose		0.358	0.36
(5) 0.18 mg. P as phosphate buffer		0.178	0.46
(6) 0.18 mg. P as phosphate buffer + glucose		0.178	0.33

i.e. during fermentation, a decrease in orthophosphate takes place inside the yeast cell, while the concentration in the surrounding fluid remains unchanged. It has been shown by Hevesy *et al.* [1937], by means of experiments with radioactive P compounds, that there is no transference of P between the yeast cell and the medium unless growth is taking place.

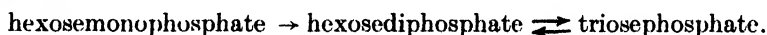
*The cycle of phosphate in glycogen and glucose fermentations in the cell*

The experiments which have been described illustrate the general principle that in the decomposition of a substrate to its normal end products by a living cell there is no unlimited accumulation of intermediate products but a constant and perhaps extremely low "working level" of such substances. For instance in fermentation by living yeast the concentration of phosphoric esters is constant and independent of the total amount of  $\text{CO}_2$  produced; this is in great contrast to fermentation in yeast juice, where in presence of excess glucose and phosphate the

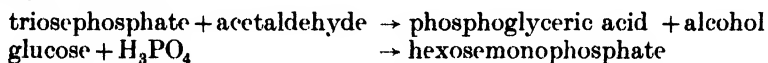
amount of esterified P found is in stoichiometric proportion to the  $\text{CO}_2$  evolved [Harden & Young, 1906].

The evidence suggests that there are two cycles with many points in common in which P is concerned in the yeast cell; firstly in the slow fermentation of glycogen present in the cell (autofermentation) and secondly in the rapid fermentation of hexoses presented to the cell. The mechanism of glycogen esterification in cell-free extracts is known to differ from that of glucose; esterification takes place in the absence of cozymase [Nilsson, 1930] and is not inhibited by fluoride [Meyerhof, 1926] or by iodoacetate [Macfarlane, 1931]. Since it is not possible to remove the preformed glycogen to ascertain the true resting level of the P the only evidence that glycogen is being esterified in the living cell is the fact that in presence of NaF esterification of inorganic P takes place. No phosphoglyceric acid was isolated from the autofermentation in presence of NaF, though it was readily obtained from an equal quantity of yeast after fermentation of sugar for the same length of time (Table I, Exp. 39).

The course of events during the fermentation of hexoses in the yeast cell is consistent with the following picture, based on the relationships between P-transference and oxidoreduction reactions which have been demonstrated in cell-free extracts [see Meyerhof *et al.* 1937; Needham & Pillai, 1937]. In the resting cell there is a small amount of hexosediphosphate (Table I) and of adenylic acid, some of which is present as adenylypyrophosphate [Wagner-Jauregg, 1936]. Part of the added hexose is therefore rapidly esterified by transference of P from adenylypyrophosphate and gives rise to triosephosphate by the reactions



The coupled reaction



begins to take place, the rate of the oxidoreduction and consequently of the esterification of inorganic P rising as the concentration of triosephosphate increases autocatalytically, until the limiting concentration of phosphoric esters is reached and the fermentation rate becomes maximal (see Table III). Once this rate is established the rate of breakdown of the phosphoric esters is in fact equal to the rate of their formation, since the level of P remains constant; when the free sugar has disappeared however it is probable that the reaction



no longer predominates in the decomposition of phosphopyruvic acid but that instead the reaction



leads to the reappearance of inorganic P in the cell.

The addition of NaF to the fermenting yeast inhibits the breakdown of phosphoglyceric acid to phosphopyruvic acid and consequently both the formation of acetaldehyde and the reformation of adenylypyrophosphate are suppressed. The ultimate effect of fluoride therefore is that the fermentation cycle is immobilized so soon as all the cozymase present has been reduced by triosephosphate, with formation of phosphoglyceric acid. Since the latter reaction is stoichiometric, the amount of phosphoglyceric acid formed in the fluoride-poisoned yeast cell should be dependent on the cozymase present, but independent of the duration of the reaction. The latter was found to be the case (Table I) while the amount of "pyro"-P found in corresponding batches of

yeast was appreciably less in the presence of fluoride than in the normal fermentation, as would be expected if reformation of adenylypyrophosphate had been inhibited. Approximately 7 mg. P per 100 g. yeast were isolated as phosphoglyceric acid, equivalent to 140 mg. cozymase. Meyerhof & Ohlmeyer [1937] estimated the cozymase content of bottom yeast as 50 mg. per 100 g., a figure based on the amount in boiled extracts, which may not represent a complete extraction. The value calculated above is only in fair agreement, but no figures are available for the actual cozymase content of the yeast used. It seems probable that in a normal fermentation this figure (7 mg. P/100 g. yeast) represents the maximum sum of the P present as 3- and 2-phosphoglyceric and phosphopyruvic acids, since for the formation of further quantities of phosphoglyceric acid the decomposition of an equivalent amount of phosphopyruvic acid is necessary.

Out of the seven phosphoric esters which are known to be intermediate products in the fermentation of sugar by yeast juice only hexosediphosphate was identified with certainty in the normal fermentation of the cell. Since the increase in organic P was of the order of 30 mg. P per 100 g. yeast of which at least 10 mg. were present as hexosediphosphate, there was an accumulation of this ester relative to the other esters. It may be surmised that this ester acts as a pressure head, maintaining by an enzymic equilibrium reaction [Meyerhof & Lohmann, 1934] the optimum supply of triosephosphate.

#### *The detection of phosphorylation in tissues*

It is clear that if the initial concentration of adenylypyrophosphate present in the tissue is so high that sufficient phosphoric ester to saturate the enzymes can be produced by direct phosphorylation of the added sugar then the esterification or even the presence of inorganic P is unnecessary. When therefore the principle is recognized that glycolysis in the living cell takes place by a cycle of P transference in which the actual amount of P involved approaches catalytic dimensions, the question of practical importance for the detection of such phosphorylation is not how much but how little P need be esterified at any one time in order to provide the concentration of phosphoric esters necessary for the maximum rate.

It is known that in certain isolated tissues [Boyland & Boyland, 1935] adenylypyrophosphate is very rapidly broken down by the enzymes present. It appears probable that if a tissue is fresh and only recently removed from its natural source of carbohydrate in the body, or if the experimental procedure necessary involves little injury, the amount of inorganic P esterified is likely to be extremely small.

This point is illustrated by observations on the orthophosphate concentration during glycolysis by a young culture of *Bact. coli*. The culture was obtained by successive tenfold dilutions with 1 % trypsin-broth of 0.5 ml. of a fresh sub-culture after 15, 7 and 6½ hr. incubation; the bacteria were separated from the broth by centrifuging 9½ hr. after the last transfer, washed twice with 0.086 % NaCl and suspended in saline. The orthophosphate content during incubation in absence and presence of glucose was then estimated. It will be seen (Table V) that the initial value of the orthophosphate and the actual decrease in presence of glucose were both very small, and that here also as in living yeast the P values increased as the glucose was consumed. The lowest amount of P actually estimated in these experiments was 0.004 mg., with an accuracy of approximately  $\pm 0.0005$  mg. P. In the absence of glucose there was a rapid increase in inorganic P, presumably by autolytic changes in an unfavourable medium; by comparison with these values the decrease during glycolysis was marked, and quite outside the experimental error.

Table V. *Inorganic P during glycolysis by Bact. coli*

2 ml. bacterial suspension (15.6 mg. dry wt. in 0.05 *M* NaHCO<sub>3</sub>, 0.015 *M* NaCl) + 1 ml. glucose or water incubated at 37°; + 3 ml. 5% trichloroacetic acid. 4 ml. samples for P estimations.

Time min.	No sugar mg. P	Sugar added	
		mg. P	mg. glucose
0	0.009	0.009	9.9
30	0.023	0.006	6.5
60	0.027	0.006	3.9
120	0.033	0.014	0.7

In experiments on the glycolysis of retina Bumm & Fehrenbach [1931] found "in glukose-freier Ringer-Lösung immer etwas mehr Phosphat abgespalten als im Ansatz mit Glukose", though the glycolysis could take place even if no detectable inorganic P were present in the tissue. The differences observed were of the order 0.002–0.004 mg. P per mg. dry wt. of retina. The glycolytic rates of yeast, *Bact. coli* and retina are high, while the observed differences of P concentration between fermenting and non-fermenting cells are of the order 1–2 mg. P/g. dry wt. If the amount of P esterified is a function of the glycolytic rate the detection of phosphorylation in tissues of low glycolytic rate will require an extremely delicate technique; especially if the tissue examined is not readily available in large amounts.

Generally speaking the rapid formation of inorganic P in an isolated tissue indicates a physiological lesion. The retina, which is often classed as having an "abnormally high" glycolytic rate, is one of the few tissues which can be detached with little injury and used entire in a manometric experiment; it is interesting to note that in the experiments of Bumm & Fehrenbach the glycolytic rate (mg. lactic acid/mg. dry wt./hr.) averaged 0.234 in 9 expts. where no inorganic P was detectable and only 0.14 in 8 expts. in which the inorganic P had increased to 0.02 mg./mg.

*The formation of glycogen in living yeast.* The occurrence of an induction period in the fermentation of sugar by living yeast has been pointed out by Willstätter & Rohdewald [1937] who found a marked increase in glycogen during this period and therefore advanced the view that glucose is not directly fermented but is first transformed into glycogen. The rapid formation of glycogen by fresh yeast has been confirmed by Goda [1938] who however does not support Willstätter & Rohdewald's hypothesis, since he found that in old yeast there was a rapid fermentation of glucose but no parallel formation of glycogen. It seems clear that glucose is directly fermented by yeast juice since the sugar is fermented more rapidly than glycogen either present in or added to the juice; it is difficult to imagine how the juice obtains this property if it is not also one of the living cell, or why glycogen newly formed from added glucose should be more rapidly fermented than glycogen preformed in the cell. Though no glycogen estimations were made in the present experiments, the experimental conditions were very similar to those of the previous workers; it seems probable therefore that the esterification of inorganic P and the formation of glycogen are contemporaneous. It was suggested by Robison [1932] that esterification of sugar might take place by a preliminary enolization and phosphorylation on carbon atom 1 of the enol sugar, followed by migration of the P group to carbon atom 6. The discovery however that the fermentation of mannose by yeast preparations may result in the almost exclusive accumulation of mannosephosphate [Jephcott & Robison, 1934] and that muscle and yeast extracts contain an enzyme, phosphohexokinase [Lohmann, 1931; Tankó & Robison, 1935] which can transform the individual

hexose-6-phosphates into an equilibrium mixture of aldose and ketose esters, indicates that enolization takes place in the phosphorylated rather than in the free sugar. Glucose-1-phosphate, which is an intermediate product in glycogen breakdown, can also be transformed enzymically into the equilibrium mixture of 6-phosphoesters [Cori *et al.* 1937]. It seems possible that the fermentable sugars are primarily phosphorylated on the reducing group, and that the observed formation of glycogen in living yeast takes place by condensation of glucose-1-phosphate with elimination of phosphate. It would be of interest to know the behaviour of fructose under similar circumstances.

#### *Non-phosphorylating glycolysis*

The demonstrated changes in P distribution during fermentation in the living yeast cell are so consistent with the theory of alcoholic fermentation developed from experiments on cell-free extracts [see Meyerhof *et al.* 1937] that there can be little doubt that the processes are analogous. Yet there are a number of differences between the cell and the cell-free extract which may be summarized as follows: fresh yeast does not ferment added glycogen or hexosediphosphate; the rate of fermentation of sugar is not increased by inorganic P, cozymase or arsenate; there is no stoichiometric relationship between the  $\text{CO}_2$  evolved and the actual decrease in inorganic P. These apparent inconsistencies may be ascribed to the fact that the cell is a well-concerted enzyme system protected by a selectively permeable membrane, so that the possession of these negative attributes by other tissues cannot furnish any evidence either for or against the existence of a phosphorylating glycolysis in these tissues. It is, however, these attributes which have been advanced as the primary experimental basis of the hypothesis of non-phosphorylating glycolysis [see Ashford & Holmes, 1929; Ashford, 1934; Needham & Nowinski, 1937; Needham *et al.* 1937]. The conclusions which have been drawn in such cases are frequently due to the expectation, either implicit or avowed, that an intact tissue or tissue slice will behave like a cell-free extract of muscle or yeast, whereas they may be expected to differ from such extracts in the same way as living yeast. For instance, the fact that the glycolysis of retina is not accelerated by phosphate or arsenate [Bumm & Fehrenbach, 1931] is more reasonably interpreted as a similarity between this tissue and living yeast than as a dissimilarity from glycolysis in yeast juice.

The demonstration of esterification during glycolysis is experimentally more difficult in tissues such as brain, kidney, tumour and chick embryo which show a rapid autolytic formation of inorganic P than in yeast where the formation of inorganic P in the resting cell is negligible in short experimental periods. In the former cases a small and limited decrease in orthophosphate due to esterification in glycolysis may be opposed to an increase due to hydrolysis by phosphatases of cell substrates which may perhaps have no connexion with the formation of lactic acid such as aminoethylphosphate or diphosphoglycerate.

Nevertheless the esterification may still be detectable; thus in the experiments of Ashford [1934] with brain tissue, the inorganic P per 100 g. tissue was 10–25 mg. less after incubation in the presence of glucose than in its absence, though both values were much greater (30–40 mg. P) than the zero value. This is indirect evidence of phosphorylation during glycolysis; the fact that the esterification was independent of the total glycolysis is consistent with the theory of a phosphorylating cycle.

The experiments of Needham *et al.* [1937] with chick embryo do not provide any evidence on this point, since no values are given for the orthophosphate in embryos incubated without glucose, a necessary control where a rapid hydrolysis

of phosphoric esters is shown to be taking place. Their values for the P distribution during glycolysis in absence and presence of fluoride show a relative increase of esterification in the latter case, particularly in the phosphoglyceric acid and hexosediphosphate fractions.

Thus in one experiment (Table XIV, *loc. cit.*) the distribution of P (in % of the acid-soluble P) was: inorganic P 48.5% and 34.7%, insoluble "resistant" ester P 0.7% and 3.6%, in absence and presence of NaF respectively; in another experiment (Table XV) inorganic P 39.3 and 27%, hexosediphosphate-P 0 and 19.8% in absence and presence of NaF. The authors' comment on these figures is that it is "at once noticeable that no accumulation of the 'hexosediphosphate' fractions has taken place...but a piling up of inorganic phosphate" at the expense of phosphagen, adenylypyrophosphate, hexosediphosphate and insoluble "resistant esters". It is true that the accumulation was only relative, but if a relative accumulation amounting to 10 or even 20% of the total acid-soluble P was insignificant, the technique was clearly inadequate for the problem.

In the interpretation of other experiments which have been adduced as evidence of non-phosphorylating glycolysis—the effect of dialysis or NaF on the glycolysis of tissue slices or brei, or the substrate-selectivity displayed by these enzyme systems—the limitations imposed by the presence of the cell membrane or by the location of the enzyme in the residual cell debris have sometimes been given insufficient weight. It is well known that the ease with which a diffusible constituent may be removed from a tissue brei or even from a cell-free extract is very variable. Thus, by dialysis against water, cozymase may be readily removed from yeast preparations but less readily from muscle extract, while for the removal of cocarboxylase alkaline washing is necessary. It cannot be assumed that because, for example, glutathione is removed by dialysis under arbitrary conditions other coenzymes which are ordinarily diffusible are also removed. The lack of response to added coenzymes in such cases may be due simply to the fact that the tissues still contain the optimum supply. On the other hand if the coenzymes have all been removed by dialysis it is necessary to restore them all to obtain reactivation; in the experiments of Needham & Lehmann [1937, p. 1230] on reactivation of dialysed chick embryo brei, for instance, in no case were adenylypyrophosphate, cozymase and  $Mg^{++}$  added simultaneously.

The difficulty in removing even inorganic P from tissue brei is illustrated in the following experiment. Weighed samples of about 1.0 g. rabbit brain were ground in a mortar with 0.5 g. silver sand and transferred quantitatively to centrifuge pots with 6 ml. borate buffer, two samples at pH 6.0 and two at pH 8.4; 2 ml.  $M$   $CaCl_2$  were added with stirring to each pot and after 20 min. at room temperature the residue was spun down and washed with the same buffer as before. All the residues were then separately extracted three times with borate buffer at pH 6.0 and finally twice with 5 ml. 5% trichloroacetic acid; the inorganic P in all the extracts was estimated. The results in Table VI show that only 80–85% of the total inorganic P present in the tissue could be extracted by buffer at pH 6.0, whether or not it had been previously rendered insoluble. It is not improbable that a residual cell structure which can prevent the outward diffusion of P can also prevent the inward diffusion of Ca. For this reason previous experiments on the immobilization of inorganic P by Ca salts in alkaline solution [e.g. Ashford & Holmes, 1929] do not give very satisfactory evidence on the participation of inorganic P in glycolysis, and moreover do not exclude the esterification of sugar by transference of P from adenylypyrophosphate.

There is other reason to suppose that there is an intracellular structure (perhaps for instance a lipoprotein complex) regulating the diffusion of sub-



Table VI. *Extraction of inorganic P from brain tissue*

g tissue	A 0.87	B 0.93 mg. P/g. tissue	C 0.92	D 0.84
Extracts 1 + 2, pH 8.4	0.024	0.014	—	—
Extracts 1 + 2, pH 6.0	—	—	0.198	0.198
Extract 3, pH 6.0	0.168	0.192	0.025	0.027
Extract 4, pH 6.0	0.058	0.054	<0.005	<0.005
Extract 5, pH 6.0	<0.005	<0.005	<0.005	<0.005
Extract 6, trichloroacetic acid	0.049	0.046	0.052	0.055
Extract 7, trichloroacetic acid	<0.005	<0.005	<0.005	<0.005
Total inorganic P extracted	0.299	0.306	0.275	0.280
% extracted in buffer	84	85	81	81

stances throughout the cytoplasm which may persist in cell fragments even if the outer membrane is disrupted. It is known for instance that if toluene is added to fresh yeast, the autofermentation rate is greatly increased [Harden, 1911]; it is difficult to account for this fact without postulating an internal change in the accessibility of the enzyme to the glycogen. It is clear that in cases where a glycolytic enzyme has not been obtained in a cell-free extract the existence of an intracellular structure affecting the accessibility of substrates has not in fact been excluded. For these reasons the view of Lehmann & Needham [1938] that "substrate-preference experiments cannot be interpreted as due to selective penetration of cell-membranes" is hardly justified till they have obtained from chick embryo a cell-free glycolysing extract which shows the same substrate-selectivity as the enzyme in the cell or cell-brei. It has been shown by Euler *et al.* [1936] and Boyland & Boyland [1938] that in brain and tumour, the selectivity exhibited by intact tissue or tissue slices to fructose and glycogen is not a property of the cell-free extract. The latter authors have pointed out that the differential inhibition of the reaction phosphoglyceric  $\rightarrow$  phosphopyruvic acid observed in brei at concentrations of NaF which do not inhibit glycolysis is probably due to the accessibility of the enzyme exposed on the injured surface both to added phosphoglyceric acid and to fluoride. The behaviour of chopped brain tissue towards glycogen, glucose and hexosediphosphate [Ashford, 1934; Ashford & Holmes, 1929] is also explicable on this basis, glucose being broken down rapidly within the cell fragment, glycogen and hexosediphosphate by enzymes exposed on the surface and partly inactivated by loss of coenzymes.

Apart from this type of experiment the most important evidence put forward in support of the hypothesis of a non-phosphorylating glycolysis is the specific inhibition of glycolysis in tumour, chick embryo etc. by *l*-glyceraldehyde. It has been shown that the phosphorylating glycolysis of dialysed muscle extracts and of washed dried yeast is also inhibited by glyceraldehyde [Adler *et al.* 1937; Boyland & Boyland, 1938]. These findings have been criticized by Lehmann & Needham [1938] on the ground that the concentration necessary for inhibition was so large that the effect is probably not the specific effect observed in tumour etc. They find it also difficult to see how the normal glycolysis of the embryo can involve the formation of hexosediphosphate, since the latter should give rise to dihydroxyacetonephosphate, and the inhibiting glyceraldehyde should in that case be removed by condensation to ketosemonophosphate. The following hypothesis explains this discrepancy and is based on the assumption that the glycolytic cycle in chick embryo is essentially similar to that of living yeast. It is known that the enzymic condensation of *d*- or *l*-glyceraldehyde with dihydroxyacetonephosphate leads to the irreversible formation of 1-fructose- and 1-sorbose-

phosphates respectively; these esters do not give rise to the equilibrium mixture of hexose-6-phosphates [Meyerhof *et al.* 1936] and take no further part in the glycolysis. Since glycolysis is inhibited by *l*- and not by *d*-glyceraldehyde, it seems probable that the fixation of triosephosphate, which can take place with either isomeride, is not the point of primary inhibition; the actual point is immaterial to the argument. The concentration of glyceraldehyde necessary for inhibition in a particular case will however be determined by the amount of triosephosphate—practically speaking by the amount of hexosediphosphate—available for fixation of the inhibitor. In the intact chick embryo the amount of hexosediphosphate present at one time will be very small; if the added glyceraldehyde can trap all the triosephosphate formed, then the glycolytic cycle will be immobilized since the further formation of adenylypyrophosphate and consequently of hexosediphosphate will be suppressed, the primary inhibition being reinforced by a secondary blockage. If however some proportion of the triosephosphate escapes fixation, the glycolytic cycle can proceed, even if diminished in extent or slowed in rate, until eventually the glyceraldehyde is decreased, by condensation, below the inhibitory concentration. The inhibition by glyceraldehyde in low concentrations is in fact transitory; with  $M/800$  in chick embryo the duration was about 1 hr.

On the other hand, both dried yeast and yeast juice normally contain hexosediphosphate; if none is present, and in the absence of adenylypyrophosphate, it is necessary to add hexosediphosphate or triosephosphate to initiate glycolysis. Moreover, yeast and muscle preparations have a relatively high esterifying power (2 g. dried yeast for instance can esterify 31 mg. P in about 10 min.) and the esterified P, mainly hexosediphosphate, remains in this type of fermentation as a by-product; it can be utilized for the removal of glyceraldehyde without affecting the subsequent fermentation. The concentration of glyceraldehyde necessary for inhibition will therefore be much higher than in a tissue of low glycolytic activity, in which a limited amount of P is taking part in a cycle.

It is premature to stress too much the lack of positive evidence of a non-phosphorylating glycolysis, for instance the extraction of an enzyme which does not require the presence of cozymase etc., since the history of the preparation of similar extracts is one of frequent trials and many failures. Nevertheless the evidence is at present negative and is based chiefly on the failure to demonstrate phenomena which are characteristic of the glycolysis of cell-free extracts rather than of cells. A critical examination of the evidence shows that in at least two of the tissues discussed, brain and retina, esterification of P during glycolysis can be inferred from the experimental data; in other cases, e.g. the inhibition by glyceraldehyde and the behaviour of tissue brei, the facts can be as consistently explained on the basis of a phosphorylating glycolysis as on any other basis. It appears that the existence of a glycolytic enzyme of a non-phosphorylating type has not yet been experimentally established.

#### SUMMARY

1. In the normal "resting" yeast cell hexosediphosphate is present in small amounts, approximately 1 mg. P/100 g. yeast.
2. During the fermentation of hexoses by living yeast the organic P present in the yeast is increased by about 30 mg. P/100 g. yeast, of which at least 10 mg. P are hexosediphosphate. This ester was identified and estimated by the formation of the crystalline phenylhydrazine salt of 6-phosphoglucosazone.

3. When the fermentation of sugars was inhibited by the addition of NaF, phosphoglyceric acid in amount equal to 7 mg. P/100 g. was isolated from the fresh yeast.

4. The observed changes in the distribution of P during fermentation in the living cell are shown to be in accord with the Embden-Meyerhof theory of alcoholic fermentation in cell-free extracts.

5. The evidence which has been put forward in favour of a theory of non-phosphorylating glycolysis in certain tissues is discussed. It is concluded that this theory is not yet experimentally justified.

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# LXXI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

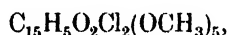
## LXI. THE MOLECULAR CONSTITUTION OF GEODIN AND ERDIN, TWO CHLORINE-CONTAINING METABOLIC PRODUCTS OF *ASPERGILLUS TERREUS* THOM. PART II. DIHYDROGEODIN AND DIHYDROERDIN AND THE SYNTHESIS OF THEIR TRIMETHYL ETHERS

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RAISTRICK & SMITH [1936] showed that a strain of *Aspergillus terreus* Thom., when grown on Czapek-Dox solution containing glucose as sole source of carbon and KCl as sole source of chlorine, produced two new metabolic products, geodin,  $C_{15}H_6O_5Cl_2(OCH_3)_2$ , and erdin,  $C_{15}H_7O_6Cl_2(OCH_3)$ , each of which on catalytic reduction gave rise to a dihydro-derivative. Later, Clutterbuck *et al.* [1937] found that both dihydrogeodin and dihydroerdin yielded the same compound on complete methylation with diazomethane, viz. a neutral substance

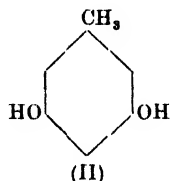
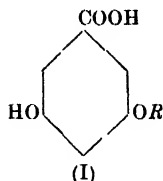


which on alkaline hydrolysis yielded a monobasic acid  $C_{15}H_6O_3Cl_2(OCH_3)_4$ , one methoxyl group having been lost. Since, as is shown later by synthesis, the latter compound contains a COOH group the formula of the neutral, fully methylated substance, may be expanded to  $C_{14}H_5OCl_2(OCH_3)_4(COOCH_3)$  and the following experiment showed that this substance is undoubtedly dihydrogeodin trimethyl ether, the COOCH<sub>3</sub> group being already present in dihydrogeodin itself but absent from dihydroerdin. When dihydrogeodin and dihydroerdin are each fully ethylated with diazoethane and the resulting neutral compounds hydrolysed by aqueous alcoholic NaOH, the same monobasic acid is obtained in each case, viz.  $C_{14}H_5OCl_2(OCH_3)(OC_2H_5)_3(COOH)$ , which still retains the methoxyl group originally present in dihydroerdin. This substance is therefore dihydroerdin triethyl ether. The corresponding monobasic acid obtained by hydrolysis of fully methylated dihydrogeodin or dihydroerdin is therefore dihydroerdin trimethyl ether and a further consequence is that dihydrogeodin is the methyl ester of dihydroerdin.

Much light has been thrown on the detailed structure of dihydrogeodin and dihydroerdin by a study of the products of reductive and hydrolytic fission.

(a) *Reductive fission.* When dihydrogeodin was refluxed with hydriodic acid (*d*, 1.7) two molecules of methyl iodide and one of CO<sub>2</sub> were evolved, and the

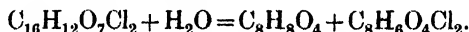
only non-volatile degradation products found were 3:5-dihydroxybenzoic acid ( $\alpha$ -resorcylic acid) (I;  $R = H$ ;  $C_7H_6O_4$ ) and orcinol (II;  $C_7H_8O_2$ ).



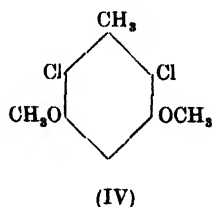
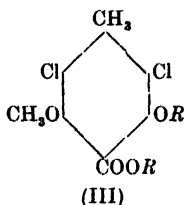
Since it is very unlikely that both these substances can arise from the same half of the molecule under the conditions of the experiment it follows that all the C atoms in dihydrogeodin are accounted for, and the action of HI may be summarized as  $C_{17}H_{14}O_7Cl_2 + 3H_2O + 4H = C_7H_8O_2 + C_7H_6O_4 + 2CH_3OH$  (as  $2CH_3I$ )  $+ CO_2 + 2HCl$ .

Dihydroerdin also yielded the same products (but with 1 mol.  $CH_3I$  in place of 2 mol.) and it is clear that the basal structure of each dihydro-compound consists of the orcinol nucleus linked in some way to the  $\alpha$ -resorcylic acid nucleus. A consideration of the expanded formula  $C_{14}H_5OCl_2(OCH_3)_4(COOCH_3)$  previously given for dihydrogeodin trimethyl ether reveals that only one oxygen atom is unaccounted for and it seemed probable therefore that this O atom is concerned in the link between the two nuclei. An obvious possibility is  $-O-$  and this possibility was confirmed by hydrolytic fission.

(b) *Hydrolytic fission.* Like many hydroxy-derivatives of benzophenone which undergo hydrolytic fission when heated with 80%  $H_2SO_4$  [cf. Graebe & Eichengrün, 1892] both dihydrogeodin and dihydroerdin are split by this reagent and give rise to the monomethyl ether of 3:5-dihydroxybenzoic acid ( $\alpha$ -resorcylic acid) (I;  $R = CH_3$ ;  $C_8H_8O_4$ ) together with an acid  $C_8H_6O_4Cl_2$ , a fission which may be represented thus for dihydroerdin:



The chlorine-containing acid was obviously a derivative of orcinol and seemed to be a dichloro-derivative of either orsellinic or *p*-orsellinic acid. On complete methylation with diazomethane it yielded methyl 2:6-dichloro-3:5-dimethoxy-*p*-toluate (III;  $R = CH_3$ ), identical in all respects with the compound prepared synthetically by the chlorination of methyl *p*-orsellinate dimethyl ether [Calam & Oxford, 1939]. The acid  $C_8H_6O_4Cl_2$  is therefore 2:6-dichloro-3:5-dihydroxy-*p*-toluic acid (III;  $R = H$ ;  $OCH_3 = OH$ ).

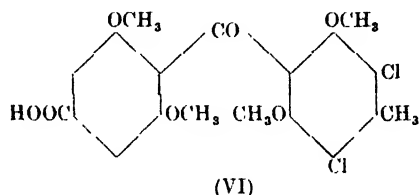
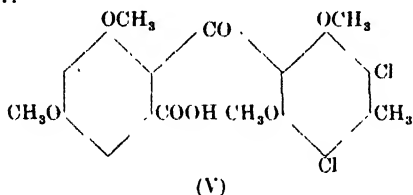


Dihydroerdin trimethyl ether also underwent a similar hydrolytic fission to yield 3:5-dimethoxybenzoic acid,  $CO_2$ , 2:6-dichloro-orcinol dimethyl ether (IV) and the monomethyl ether of dichloro-*p*-orsellinic acid (III;  $R = H$ ).

Compound (IV) was also obtained in quite another way. The thermal degradation of geodin and erdin, as well as of dihydroerdin, at  $250^\circ$ , yielded a sublimate consisting essentially of a phenolic substance  $C_7H_6O_2Cl_2$ , 2:6-dichloro-orcinol, which on methylation yielded IV ( $C_9H_{10}O_2Cl_2$ ) identical in all respects

with the dimethyl ether of 2:6-dichloro-orcinol prepared synthetically [Calam & Oxford, 1939].

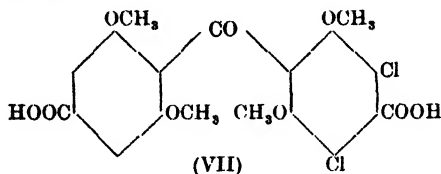
The above evidence strongly suggests that dihydrogeodin and dihydroerdin are derivatives of benzophenone and, since two COOH groups appear in the fission products, one must be concerned in the union between the two benzene rings, dihydroerdin trimethyl ether being only a monobasic acid. The only possible links between the two rings are therefore  $\text{—CO—}$  and  $\text{—CO.CH}_2\text{—}$ . The latter must be incorrect since dihydroerdin trimethyl ether, when oxidized by boiling alkaline  $\text{KMnO}_4$ , forms a dibasic acid with the same number of C atoms but with two O atoms more and two H atoms less than the starting material. This can be explained only by the oxidation of  $\text{CH}_3$  to COOH, hence the  $\text{CH}_3$  group of the dichloro-orcinol half of the molecule must exist as such in the dihydro-compounds. The only possible link between the two benzene nuclei is therefore  $\text{—CO—}$ , and the failure of all our attempts to prepare derivatives of this carbonyl group is doubtless due to the steric effects of the adjacent  $\text{OCH}_3$  groups. There are thus two possible structures for dihydroerdin trimethyl ether, viz.:



for the free COOH group in this compound must be attached to the non-chlorinated benzene ring, there being no free position in the other ring. Each of these structures will yield 3:5-dimethoxybenzoic acid and 2:6-dichloro-3:5-dimethoxy-*p*-toluic acid or its degradation products on hydrolytic fission. Since no dimethoxy-phthalic or -terephthalic acid is formed in the fission, the evidence so far presented does not enable a decision to be made between (V) and (VI). Actually (V) is almost certainly correct for the following reasons.

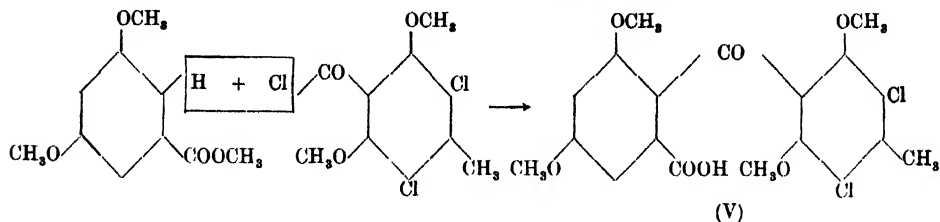
(1) Dihydroerdin trimethyl ether can be acetylated to yield a neutral monoacetyl derivative. This may be explained on (V) (a  $\gamma$ -keto-acid behaving as a  $\gamma$ -hydroxy- $\gamma$ -lactone) for which there are many analogies including that of benzophenone-2-carboxylic acid [von Pechmann, 1881; Graebe, 1900], but cannot be explained convincingly on (VI).

(2) The dibasic acid obtained by alkaline  $\text{KMnO}_4$  oxidation of dihydroerdin trimethyl ether must be (VII) if derived from (VI). But such a structure is



quite symmetrical about the  $\text{—CO—}$  link (except for the two Cl atoms, whose effect on the reactivity of the molecule is probably very small in comparison with that of the four methoxyl groups), and ought to yield both 3:5-dimethoxybenzoic acid and 2:6-dimethoxyterephthalic acid on hydrolytic fission. Actually 3:5-dimethoxybenzoic acid and 3:5-dichloro-2:6-dimethoxyterephthalic acid were the only hydrolytic products isolated.

(3) Dihydroerdin trimethyl ether has been synthesized by an application of the Friedel-Crafts reaction, 2:6-dichloro-3:5-dimethoxy-*p*-toluyl chloride being condensed directly with methyl 3:5-dimethoxybenzoate. From the known



behaviour of resorcinol dimethyl ether towards acid chlorides in the Friedel-Crafts reaction it might be predicted with confidence that in the above more complex instance the entering group would also occupy the position *para* to one OCH<sub>3</sub> group and *ortho* to the other, to the almost total exclusion of the position *ortho* to each OCH<sub>3</sub> group. The presence of COOCH<sub>3</sub> *meta* to each OCH<sub>3</sub> would tend to strengthen this preference. Thus Jones & Robinson [1917], discussing the influence of a negative group on the directing effect of a positive group in benzene substitution, conclude "there is evidence that it is not merely *ortho* substitution which is favoured but that it is the particular *ortho* position situated between the positive and negative groups". There can be little doubt therefore that dihydroerdin trimethyl ether is 3':5'-dichloro-4:6:2':6'-tetramethoxy-4'-methylbenzophenone-2-carboxylic acid (V). Since the hydrolytic fission of dihydroerdin itself yields the monomethyl ether of  $\alpha$ -resorcylic acid, dihydroerdin is evidently either 3':5'-dichloro-6:2':6'-trihydroxy-4-methoxy- or 3':5'-dichloro-4:2':6'-trihydroxy-6-methoxy-4'-methylbenzophenone-2-carboxylic acid, but the evidence so far adduced offers no clue as to which structure is the correct one.

The methylation of synthetic dihydroerdin trimethyl ether with diazomethane yielded its methyl ester identical in all respects with dihydrogeodin trimethyl ether obtained by the complete methylation of dihydrogeodin, prepared by the catalytic reduction of geodin from *A. terreus*. Dihydrogeodin trimethyl ether is thus methyl 3':5'-dichloro-4:6:2':6'-tetramethoxy-4'-methylbenzophenone-2-carboxylate (V; COOH = COOCH<sub>3</sub>).

#### EXPERIMENTAL

*Equivalents of dihydrogeodin and dihydroerdin.* The compounds were dissolved in aqueous ethanol and titrated directly with 0.1 *N* NaOH, with phenolphthalein as indicator. *Dihydrogeodin*: Found, 322. C<sub>17</sub>H<sub>14</sub>O<sub>7</sub>Cl<sub>2</sub> requires 401 (as monobasic acid) and 200.5 (as dibasic acid). *Dihydroerdin*: Found, 201. C<sub>16</sub>H<sub>12</sub>O<sub>7</sub>Cl<sub>2</sub> requires 193.5 (as dibasic acid).

Dihydroerdin is thus clearly dibasic and dihydrogeodin more nearly monobasic than dibasic.

#### *Ethylation of dihydrogeodin and dihydroerdin and subsequent hydrolysis to dihydroerdin triethyl ether*

Dihydrogeodin (0.45 g.) was treated with excess of ethereal diazoethane for 30 min. The solvent was removed leaving a reddish gum (0.64 g.) which was hydrolysed by refluxing with ethanol (50 ml.) and *N* NaOH (25 ml.) for 1 hr. After removal of the ethanol *in vacuo* and acidification, the precipitated acid

was collected (0.59 g.; M.P. 185°) and crystallized from benzene-light petroleum in colourless rectangular prisms, M.P. 211–213°. (Found: C, 55.95, 56.0; H, 5.2, 5.0;  $\text{CH}_3\text{O}$ , 24.9, 25.1%.  $\text{C}_{22}\text{H}_{24}\text{O}_7\text{Cl}_2$  (dihydroerdin triethyl ether) requires C, 56.05; H, 5.1;  $\text{CH}_3\text{O} + 3\text{C}_2\text{H}_5\text{O}$  (calc. as  $4\text{CH}_3\text{O}$ ), 26.3%.  $\text{C}_{21}\text{H}_{22}\text{O}_7\text{Cl}_2$  (diethyl-dihydrogeodin) requires C, 55.1; H, 4.85;  $2\text{CH}_3\text{O} + 2\text{C}_2\text{H}_5\text{O}$  (calc. as  $4\text{CH}_3\text{O}$ ), 27.1%.) Dihydroerdin, treated in exactly the same way, gave identical rectangular prisms, M.P. 211–212°, alone or mixed with the acid obtained from dihydrogeodin. (Found: C, 56.2; H, 5.1; Cl, 14.8, 14.7;  $\text{CH}_3\text{O}$ , 25.1, 25.5%. Equiv. 486.  $\text{C}_{22}\text{H}_{24}\text{O}_7\text{Cl}_2$  requires C, 56.05; H, 5.1; Cl, 15.05;  $\text{CH}_3\text{O} + 3\text{C}_2\text{H}_5\text{O}$  (calc. as  $4\text{CH}_3\text{O}$ ), 26.3%. Equiv. 471.)

*Acetyl derivative of dihydroerdin trimethyl ether*

Dihydroerdin trimethyl ether (0.5 g.) was heated for 30 min. at 150–160° with anhydrous sodium acetate (1 g.), acetic anhydride (2 ml.) and glacial acetic acid (3 ml.). Water was then cautiously added to the cooled mixture, and the precipitated gum hardened after standing for several hours. The solid product was crystallized from light petroleum (B.P. 80–100°) and separated in rosettes of prisms (0.13 g.). Unlike the starting material it was not completely soluble in dilute  $\text{NaHCO}_3$  solution. The  $\text{NaHCO}_3$ -insoluble portion (0.03 g.) was crystallized from  $\text{CCl}_4$ -light petroleum and formed compact square prisms, M.P. 208–210°. (Found: C, 53.5, 53.5; H, 4.15, 4.35; Cl, 15.1; hydrolysable  $\text{CH}_3\text{CO}$ , 8.6;  $\text{CH}_3\text{O}$ , 24.85%.  $\text{C}_{21}\text{H}_{20}\text{O}_8\text{Cl}_2$  requires C, 53.5; H, 4.3; Cl, 15.05;  $\text{CH}_3\text{CO}$ , 9.1;  $4\text{CH}_3\text{O}$ , 26.3%.) The acetyl derivative is quite insoluble in cold caustic soda solution and dissolves only slowly on heating.

*Hydrolysis.* 0.02382 g. in 10 ml. EtOH + 5 ml.  $\text{H}_2\text{O}$  required only 0.004 ml.  $N/10$  NaOH for neutralization to phenolphthalein. 1.891 ml.  $N/10$  NaOH were then added and the corked flask kept at 37° overnight. Required: 1.034 ml.  $N/10$   $\text{H}_2\text{SO}_4$  for back titration. Hence equiv. = 278.  $\text{C}_{21}\text{H}_{20}\text{O}_8\text{Cl}_2$  titrating as a dibasic acid requires equiv. = 235.5.

*Oxidation of dihydroerdin trimethyl ether to a dibasic acid containing the same number of carbon atoms*

Dihydroerdin trimethyl ether,  $\text{C}_{19}\text{H}_{18}\text{O}_7\text{Cl}_2$  (0.5 g.), was dissolved in 0.1  $N$  NaOH (30 ml.) and the solution refluxed with slow dropwise addition of 5% aqueous  $\text{KMnO}_4$ . A stable purple colour was reached after 30 ml. of the latter had been added during 2½ hr. The liquid was cooled, decolorized with  $\text{SO}_2$  and acidified with dilute  $\text{H}_2\text{SO}_4$ . The precipitated acid separated as colourless needles (0.33 g., M.P. 229–231°), raised to 233–235° by recrystallization from hot water. (Found: C, 49.85; H, 3.6; Cl, 15.7, 15.25;  $\text{CH}_3\text{O}$ , 27.3%. Equiv. 227.  $\text{C}_{19}\text{H}_{16}\text{O}_9\text{Cl}_2$  requires C, 49.7; H, 3.5; Cl, 15.45;  $4\text{CH}_3\text{O}$ , 27.0%. Equiv. 229.5 (titrating as a dibasic acid).)

Several attempts were made partially to methylate dihydroerdin with diazomethane and then to oxidize away the incompletely methylated parts of the molecule in the hope of isolating a recognizable dimethoxy-phthalic or -terephthalic acid, but even the product obtained by the addition of 2 mol. of diazomethane to dihydrogeodin was completely destroyed by  $\text{KMnO}_4$  both in aqueous alkaline and in acetone solutions.

*Reductive fission experiments*

*A. Dihydrogeodin.* Dihydrogeodin (0.8487 g.) was heated with HI (15 ml.;  $d$ , 1.7) in a Zeisel apparatus in a stream of dry  $\text{CO}_2$ -free nitrogen, the bath temperature being maintained at 140–150°. The issuing gases were passed



through bubblers containing alcoholic  $\text{AgNO}_3$  and standard baryta solution respectively. The  $\text{AgI}$  produced in the former amounted to 0.9739 g. (calc. for  $2\text{CH}_3\text{O}$ , 0.9928 g.) whilst the  $\text{CO}_2$  absorbed in the latter was equivalent to 44.05 ml. 0.1  $N$  (calc. for 1 mol.  $\text{CO}_2$ , 42.3 ml. 0.1  $N$ ). The residual  $\text{HI}$  solution was washed into a continuous extractor and extracted with ether for 8 hr. The material left after removal of the solvent was dissolved in water and heated on the steam bath with red phosphorus to remove iodine, filtered and re-extracted with ether. The dried solid obtained from the final extract (0.635 g.; calc. 0.627 g.) was fractionally sublimed in a high vacuum, first at  $80^\circ$  to yield sublimate I (0.22 g.) and then at  $160^\circ$  to yield sublimate II (0.25 g.).

*Sublimate I*, after re-sublimation, was free from chlorine and melted at  $108^\circ$  alone or mixed with authentic anhydrous orcinol (m.p.  $108^\circ$ ). In aqueous alcoholic solution it gave a violet colour with  $\text{FeCl}_3$ , identical with that given by orcinol. (Found: C, 68.0, 67.7; H, 6.5, 6.4%.  $\text{C}_7\text{H}_8\text{O}_2$  requires C, 67.7; H, 6.5%.)

*Sublimate II*, after re-sublimation, was free from chlorine and melted at  $236^\circ$ , alone or mixed with authentic  $\alpha$ -resorecylic acid (m.p.  $236^\circ$ ) obtained by the  $\text{HI}$  demethylation of 3:5-dimethoxybenzoic acid. (Found: C, 54.8, 54.55; H, 4.1, 3.9%.  $\text{C}_7\text{H}_6\text{O}_4$  requires C, 54.5; H, 3.9%.) On methylation with diazomethane and hydrolysis of the resulting methyl ester, 3:5-dimethoxybenzoic acid was obtained, m.p.  $181$ – $182^\circ$ , alone or mixed with the authentic acid prepared by the hot alkaline  $\text{KMnO}_4$  oxidation of orcinol dimethyl ether.

B. *Dihydroerodin*. Dihydroerodin (0.873 g.) under the same conditions gave  $\text{CO}_2$  equivalent to 44.5 ml. 0.1  $N$  (calc. for 1 mol.  $\text{CO}_2$ , 45.5 ml.) and  $\text{CH}_3\text{I}$  (as  $\text{AgI}$ ) equivalent to one  $\text{CH}_3\text{O}$  group. From the  $\text{HI}$  was recovered 0.68 g. of crude solid material (calc. 0.64 g.) which was fractionally sublimed as before to yield orcinol (0.15 g.) and a small amount of  $\alpha$ -resorecylic acid.

#### *Hydrolytic fission experiments*

A. *Dihydrogeodin*. Dihydrogeodin (1 g.) was gradually heated in a small flask on a wire gauze with a mixture of conc.  $\text{H}_2\text{SO}_4$  (20 ml.) and water (10 ml.) with a thermometer in the liquid. At  $120^\circ$  the colour of the liquid suddenly became lighter, the heating was immediately stopped and the flask allowed to cool. Water (100 ml.) was then cautiously added and the precipitated solid collected, washed and dried (0.5 g.). The filtrate, after ether extraction, yielded a similar solid (0.5 g.). The combined products were repeatedly and fractionally sublimed in a high vacuum giving two main fractions.

*Sublimate I* sublimed at  $110$ – $120^\circ$ , melted at  $214^\circ$  and gave an intense blue colour with  $\text{FeCl}_3$ . (Found: C, 40.7, 40.6; H, 2.7, 2.8; Cl, 29.4, 29.6%.  $\text{C}_8\text{H}_6\text{O}_4\text{Cl}_2$  requires C, 40.5; H, 2.55; Cl, 30.0%.) It was shown to be 2:6-dichloro-3:5-dihydroxy-*p*-toluic acid by complete methylation with diazomethane when methyl 2:6-dichloro-3:5-dimethoxy-*p*-toluate, m.p.  $85$ – $86^\circ$ , was obtained, identical in all respects (crystalline form; mixed m.p.; solubilities) with the ester prepared synthetically [Calam & Oxford, 1939]. (Found: C, 47.7; H, 4.3; Cl, 25.2, 25.2;  $\text{CH}_3\text{O}$ , 33.7%.  $\text{C}_{11}\text{H}_{12}\text{O}_4\text{Cl}_2$  requires C, 47.3; H, 4.3; Cl, 25.4;  $3\text{CH}_3\text{O}$ , 33.3%.) The above fission product, when heated above its m.p., lost  $\text{CO}_2$  to yield 2:6-dichloro-orcinol, m.p.  $164^\circ$ , identical with the chlorophenol obtained by thermal degradation of erdin (see later).

*Sublimate II* sublimed at  $120$ – $140^\circ$ , and was finally obtained pure only after repeated fractional sublimation. It melted at  $201^\circ$  and did not contain chlorine, nor did it give any coloration with  $\text{FeCl}_3$ . It was soluble in  $\text{NaHCO}_3$  solution with evolution of  $\text{CO}_2$ . (Found: C, 56.8; H, 4.9;  $\text{CH}_3\text{O}$ , 17.2%.  $\text{C}_8\text{H}_8\text{O}_4$  requires

C, 57.15; H, 4.8;  $1\text{CH}_3\text{O}$ , 18.4 %.) It was shown to be the monomethyl ether of  $\alpha$ -resorcylic acid (Mauthner [1927] gives m.p. 202–203°) by methylating it with diazomethane followed by hydrolysis of the resulting ester with aqueous alcoholic NaOH to yield 3:5-dimethoxybenzoic acid, m.p. 181–182°, alone or mixed with an authentic specimen.

B. *Dihydroerdin*. A more efficient and rapid method of separation of the fission products was devised in this case, advantage being taken of the sparing solubility of the sodium salt of the monomethyl ether of  $\alpha$ -resorcylic acid in an excess of  $\text{NaHCO}_3$  solution. Dihydroerdin (1.207 g.) and a mixture of conc.  $\text{H}_2\text{SO}_4$  (22 ml.) and water (11 ml.) were heated together in a stream of  $\text{CO}_2$ -free nitrogen and the issuing gases passed through a bubbler containing standard baryta solution. The reaction flask was slowly heated in an oil bath and when the latter had reached 125° the red solution suddenly became nearly colourless, with simultaneous separation of a solid. The oil bath was at once removed and the reaction mixture cooled. The  $\text{CO}_2$  evolved was equivalent to only 3.5 ml. 0.1 *N* NaOH. Water (65 ml.) was added to the reaction mixture and the precipitated solid was later collected, washed and dried. The crude product (1.02 g.) was shaken with a slight excess of saturated  $\text{NaHCO}_3$  solution to neutralize it, and again filtered.

The filtrate on acidification gave an acid (0.42 g.), m.p. 200–205°, raised to 214° by a single sublimation in a high vacuum. This acid was identical with the acid, m.p. 214°, obtained in the hydrolytic fission of dihydrogeodin, and was therefore 2:6-dichloro-3:5-dihydroxy-*p*-toluic acid. On methylation with diazomethane it yielded methyl 2:6-dichloro-3:5-dimethoxy-*p*-toluate identical in all respects with an authentic specimen of the ester prepared synthetically.

The  $\text{NaHCO}_3$ -insoluble residue was treated with dilute  $\text{H}_2\text{SO}_4$  and extracted with ether, the extract being then shaken with dilute aqueous  $\text{NaHCO}_3$ . The non-acidic substance remaining in the ether (0.03 g.; calc. from  $\text{CO}_2$  produced in the fission, 0.034 g.) gave on sublimation a product of m.p. 165°, identical with 2:6-dichloro-*orcinol* obtained by thermal degradation of erdin (see later). The above bicarbonate extract was acidified and extracted with ether to yield a product (0.54 g.) which, when crystallized once from hot water, had m.p. 197–197.5°, and was free from chlorine. It was identical in all respects with the monomethyl ether of  $\alpha$ -resorcylic acid obtained in the hydrolytic fission of dihydrogeodin. (Found: C, 56.7, 56.7; H, 4.95, 4.7;  $\text{CH}_3\text{O}$ , 17.15, 17.35 %.  $\text{C}_8\text{H}_8\text{O}_4$  requires C, 57.15; H, 4.8;  $1\text{CH}_3\text{O}$ , 18.4 %.)

C. *Dihydrogeodin trimethyl ether*. The ether (1.0 g.) was heated with a mixture of conc.  $\text{H}_2\text{SO}_4$  (20 ml.) and water (10 ml.) to 120° when the colour suddenly changed from deep orange to light brown. After cooling, water (40 ml.) was added and the precipitated solid (1.15 g.) collected later, washed and dried. It was covered with a slight excess of saturated  $\text{NaHCO}_3$  solution, and, after standing for a while, filtered.

The filtrate, on acidification, yielded a crude product (0.59 g.) which after one recrystallization from benzene gave rather impure 3:5-dimethoxybenzoic acid (0.24 g.) (see below). The benzene mother liquor, on evaporation, yielded a different substance (0.17 g.) which was purified by sublimation followed by crystallization from aqueous alcohol. It formed hair-like needles, m.p. 201°, which contained chlorine and gave a violet colour with  $\text{FeCl}_3$ . (Found: C, 43.1, 43.1; H, 3.2, 3.15;  $\text{CH}_3\text{O}$ , 12.2 %.  $\text{C}_9\text{H}_8\text{O}_4\text{Cl}_2$  requires C, 43.0; H, 3.2;  $1\text{CH}_3\text{O}$ , 12.35 %.) That it was 2:6-dichloro-3-hydroxy-5-methoxy-*p*-toluic acid (III,  $R=\text{H}$ ) was shown by its identity with this substance prepared synthetically (Calam & Oxford [1939] who quote m.p. 202–203°) and by the fact that it

yielded methyl 2:6-dichloro-3:5-dimethoxy-*p*-toluate (III;  $R = \text{CH}_3$ ) on methylation with diazomethane.

The  $\text{NaHCO}_3$ -insoluble portion mentioned above was treated with 0.2 *N*  $\text{NaOH}$  (10 ml.), and the undissolved residue (0.12 g.) was recrystallized from light petroleum to yield prisms, m.p. 131–133°, not depressed on admixture with an authentic specimen of 2:6-dichloro-orcinol dimethyl ether (IV). The alkali-soluble material was recovered (0.25 g.) and proved to be chiefly 3:5-dimethoxybenzoic acid (0.49 g. in all). The combined specimens were recrystallized from hot water to yield needles, m.p. 181–182°, not depressed on admixture with an authentic specimen of 3:5-dimethoxybenzoic acid.

D. *The dibasic acid  $\text{C}_{19}\text{H}_{16}\text{O}_9\text{Cl}_2$  obtained by oxidation of dihydroerdin trimethyl ether.* The acid (0.85 g.) was heated with conc.  $\text{H}_2\text{SO}_4$  (17 ml.) and water (8 ml.) to 110° when the colour changed from orange to light brown. After cooling, an equal volume of water was added and the precipitated solid collected after an hour (0.55 g.). It gave only a faint violet coloration with  $\text{FeCl}_3$ .

After solution in dilute aqueous  $\text{NaHCO}_3$  and filtration from a little tarry material it was reprecipitated (0.25 g.) and purified, by sublimation in a high vacuum at 100°, to yield chlorine-free 3:5-dimethoxybenzoic acid, m.p. and mixed m.p. 183°. (Found:  $\text{CH}_3\text{O}$ , 33.1%.  $\text{C}_9\text{H}_{10}\text{O}_4$  requires 2( $\text{H}_3\text{O}$ , 34.1%.)

The original acid mother liquor from the fission and the filtrate from the above crude dimethoxybenzoic acid were extracted with ether and the combined crude products (0.44 g.) crystallized from ethyl acetate-light petroleum to give colourless needles, m.p. 236–238°, alone or mixed with authentic 3:5-dichloro-2:6-dimethoxyterephthalic acid, m.p. 235–237° [Calam & Oxford, 1939]. (Found: C, 40.1; H, 2.8; Cl, 24.7%. Equiv. 143.5.  $\text{C}_{10}\text{H}_8\text{O}_6\text{Cl}_2$  requires C, 40.7; H, 2.7; Cl, 24.1%. Equiv. 147.5.)

*Thermal degradation of geodin, erdin and dihydroerdin with production of 2:6-dichloro-orcinol*

Geodin, erdin and dihydroerdin all give the same volatile product on thermal degradation. The following is a typical experiment: erdin (1 g.) was heated at 250° for 1½ hr. in a long boiling tube fitted with a small condenser, when long, colourless needles sublimed (0.15 g.). This product, after resublimation, melted at 164°. (Found: C, 43.9, 44.1; H, 3.1, 3.2; Cl, 36.0, 36.3%; mol. wt. (cryoscopic in camphor), 193, 189.  $\text{C}_7\text{H}_6\text{O}_2\text{Cl}_2$  requires C, 43.5; H, 3.1; Cl, 36.75%; mol. wt. 193.) It was soluble in caustic soda solution and contained two active H atoms as determined by the Zerewitinoff method (Roth). (Found: 1.8 active H atoms both in anisole at 19° and in pyridine at 95°.) It also contained one Me side chain, as determined by Kuhn-Roth oxidation. (Found: 0.947, 0.943 mol. of  $\text{CH}_3\text{COOH}$  per mol. of  $\text{C}_7\text{H}_6\text{O}_2\text{Cl}_2$ ) and was therefore undoubtedly a dichlorodihydroxy-methylbenzene. That it was 2:6-dichloro-orcinol was proved by methylation with excess of ethereal diazomethane, when 2:6-dichloro-orcinol dimethyl ether (IV), m.p. 129°, was obtained, identical in all respects, including mixed m.p., with the authentic compound, m.p. 133–134°, prepared synthetically [Calam & Oxford, 1939]. (Found: C, 49.0, 48.8; H, 4.4, 4.55; Cl, 31.6, 31.6%.  $\text{C}_9\text{H}_{10}\text{O}_2\text{Cl}_2$  requires C, 48.8; H, 4.6; Cl, 32.1%.) Dihydroerdin behaved very similarly to erdin, but with geodin the yield was smaller and the sublimate less pure.

*Syntheses*

A. *Dihydroerdin trimethyl ether. (3':5'-Dichloro-4:6:2':6'-tetramethoxy-4'-methylbenzophenone-2-carboxylic acid (V).)* A mixture of 2:6-dichloro-3:5-dimethoxy-*p*-toluic acid (5 g.) and thionyl chloride (7 ml.) was kept for an hour,

after which excess of the latter was removed under reduced pressure. The resulting crude acid chloride, methyl 3:5-dimethoxybenzoate (5.5 g.) and powdered anhydrous aluminium chloride (5 g.), were placed in a long-necked round-bottomed flask, which, after displacement of the air by dry HCl gas, was fitted with a cork bearing a  $\text{CaCl}_2$ -tube. The mixture was heated on the steam bath for 10 min. until a homogeneous reddish-brown semi-solid mass had formed and was left at room temperature for 2 days. Water was added and unchanged methyl 3:5-dimethoxybenzoate removed by distillation in steam. The liquid was decanted from the residual gum which was methylated by treatment with acetone (30 ml.), methyl sulphate (10 ml.) and 2N NaOH (80 ml. in 8 successive portions of 10 ml.) with continuous shaking. The semi-solid product was washed by decantation several times and hydrolysed by refluxing with ethanol (50 ml.) and N NaOH (50 ml.) for 1 hr. After addition of water, cooling and filtration from a little oily impurity, the crude acid was precipitated by acidification (3.7 g.). After one crystallization from  $\text{CCl}_4$ -light petroleum it melted at  $149\text{--}159^\circ$  (1.6 g.) and from the mother liquor 1.4 g. of nearly pure 2:6-dichloro-3:5-dimethoxy-*p*-toluic acid were recovered, M.P.  $107\text{--}112^\circ$ . The product, M.P.  $149\text{--}159^\circ$ , was further purified by repeated crystallization from  $\text{CCl}_4$  to yield colourless laminae, M.P.  $177\text{--}179^\circ$ , readily soluble in dilute aqueous  $\text{NaHCO}_3$ . (Found: C, 52.65; H, 4.1; Cl, 16.6, 16.8;  $\text{CH}_3\text{O}$ , 28.6, 28.8%.  $\text{C}_{19}\text{H}_{18}\text{O}_7\text{Cl}_2$  requires C, 53.1; H, 4.2; Cl, 16.55;  $4\text{CH}_3\text{O}$ , 28.9%.) Clutterbuck *et al.* [1937] quote  $168^\circ$  for the M.P. of natural dihydroerdin trimethyl ether, but the M.P. of their material was raised after several recrystallizations from  $\text{CCl}_4$  to  $177\text{--}179^\circ$  unaltered by admixture with the above synthetic product of the same M.P.

B. *Dihydrogeodin trimethyl ether*. (Methyl 3':5'-dichloro-4:6:2':6'-tetramethoxy-4'-methylbenzophenone-2-carboxylate (V;  $\text{COOH} = \text{COOCH}_3$ )). The above synthetic dihydroerdin trimethyl ether (M.P.  $177\text{--}179^\circ$ ; 0.1 g.) was treated with excess of ethereal diazomethane, and the resulting ester crystallized from aqueous methyl alcohol to yield colourless needles, M.P.  $110\text{--}112^\circ$ , alone or mixed with dihydrogeodin trimethyl ether (M.P.  $108^\circ$ ) prepared from natural dihydrogeodin. (Found: C, 53.65; H, 4.2; Cl, 16.05, 15.8;  $\text{CH}_3\text{O}$ , 34.3%.  $\text{C}_{20}\text{H}_{20}\text{O}_7\text{Cl}_2$  requires C, 54.15; H, 4.55; Cl, 16.0;  $5\text{CH}_3\text{O}$ , 35.0%.)

C. *Decarboxydihydroerdin trimethyl ether*. (3:5-Dichloro-2:6:2':4'-tetramethoxy-4-methylbenzophenone.) Resorcinol dimethyl ether (5 g.) and anhydrous aluminium chloride (5 g.) were added to crude 2:6-dichloro-3:5-dimethoxy-*p*-toluyl chloride (5 g.) prepared as before (see above). The mixture was cooled, the air displaced by dry HCl gas, and the flask fitted with a cork bearing a  $\text{CaCl}_2$ -tube. After heating on the water bath until a homogeneous dark red mass had formed, the flask was kept for 2 days. The reaction mixture was decomposed with ice-water and the unchanged resorcinol dimethyl ether removed by steam distillation. The residual solid was taken up in ether, the extract washed with dilute aqueous  $\text{NaHCO}_3$ , and the solvent removed to yield a crude product (4.2 g.) which was dissolved in acetone (20 ml.) and treated with dimethyl sulphate (10 ml.) and 2N NaOH (80 ml. in ten successive lots) with continuous shaking. The solid product (4 g.) was crystallized from aqueous methyl alcohol to yield colourless parallelepipeds, M.P.  $94\text{--}95^\circ$ . (Found: C, 56.4, 56.3; H, 4.8, 4.7; Cl, 18.4;  $\text{CH}_3\text{O}$ , 31.5%.  $\text{C}_{18}\text{H}_{18}\text{O}_5\text{Cl}_2$  requires C, 56.1; H, 4.7; Cl, 18.4;  $4\text{CH}_3\text{O}$ , 32.2%.)

Many attempts were made to decarboxylate dihydroerdin trimethyl ether in the hope of obtaining the above substituted benzophenone. These included heating in a sealed tube with water at  $140^\circ$  and with water and Cu bronze at

250°, heating the ether in the dry state in  $N_2$  at 250–300° (practically no  $CO_2$  evolved) and heating with quinoline and Cu chromite. All were fruitless, the material being either completely broken down or recovered unchanged.

D. *Homodecarboxydihydroerдин trimethyl ether*. (3:5-Dichloro-2:6:2':4'-tetramethoxy-4:6'-dimethylbenzophenone.) The synthesis was carried out exactly as in the previous section save that orcinol dimethyl ether (5 g.) was used in place of resorcinol dimethyl ether. The crude product after methylation (3.5 g.) was purified by repeated crystallization from aqueous methyl alcohol. Large, colourless rectangular prisms, m.p. 105–106°. (Found: C, 57.1, 57.2; H, 5.1, 5.0; Cl, 17.95;  $CH_3O$ , 31.3, 31.15 %.  $C_{19}H_{20}O_5Cl_2$  requires C, 57.1; H, 5.05; Cl, 17.8;  $4CH_3O$ , 31.1 %.)

In this preparation the  $NaHCO_3$  extract of the ethereal solution of the crude product (after removal of unchanged orcinol dimethyl ether) yielded 2:6-dichloro-3:5-dihydroxy-p-toluic acid on acidification; m.p. 214° after two sublimations in a high vacuum. This constitutes a synthesis of the chloro-acid obtained by the hydrolytic fission of dihydrogeodin and dihydroerдин (pp. 584–5).

Several attempts were made to oxidize both  $CH_3$  groups in the above substituted benzophenone to  $COOH$  in the hope of obtaining the dibasic acid previously obtained by the oxidation of dihydroerдин trimethyl ether (p. 583). These included the action of  $KMnO_4$  in alkaline and in acid solution;  $CrO_3$  in acetic acid-acetic anhydride solution;  $K_2Cr_2O_7$  in  $H_2SO_4$  solution; and 25 % aqueous  $HNO_3$ . All were fruitless, the compound being either completely destroyed or recovered unchanged.

#### SUMMARY

Geodin ( $C_{15}H_6O_5Cl_2(OCH_3)_2$ ) and erдин ( $C_{15}H_7O_6Cl_2(OCH_3)$ ), metabolic products of *Aspergillus terreus* Thom, each give rise to a dihydro-derivative on catalytic reduction, dihydrogeodin being the methyl ester of dihydroerдин. As a result of analytical and synthetic methods dihydrogeodin trimethyl ether,  $C_{14}H_5OCl_2(OCH_3)_4COOCH_3$ , has been shown to be methyl 3':5'-dichloro-4:6:2':6'-tetramethoxy-4'-methylbenzophenone-2-carboxylate and dihydroerдин trimethyl ether,  $C_{14}H_5OCl_2(OCH_3)_4COOH$ , to be 3':5'-dichloro-4:6:2':6'-tetramethoxy-4'-methylbenzophenone-2-carboxylic acid.

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# LXXII. CRYSTALLINE ESTERS OF VITAMIN A

## I. PREPARATION AND PROPERTIES

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### PART I

THE preparation of the crystalline 2-naphthoate and anthraquinone-2-carboxylate of vitamin A has been reported by Hamano [1935; 1937] who used as starting material the non-saponifiable matter from fish liver oils which are exceptionally rich in this vitamin. Hamano found that these esters were biologically active but did not submit them to a quantitative animal assay. The work described in this paper was undertaken in order to find out whether crystalline esters could be prepared from vitamin A obtained from commercial oils and to compare their biological activity with that of the International Standard.

By molecular distillation of vitamin A alcohol, fractions with coefficients of absorption ( $E_{1\text{cm}}^{1\%}$  328  $m\mu$ ) exceeding 1600 were obtained. These were used for the preparation of several synthetic esters of which the 2-naphthoate and anthraquinone-2-carboxylate alone were obtained crystalline. The properties of the crystalline esters made in this way resembled those of the specimens prepared by Hamano, the anthraquinone-2-carboxylate being obtained in both the yellow and the red forms described by this author.

The ultraviolet absorption of the esters has been examined and from the results it has been deduced that the vitamin A alcohol combined in these substances has  $E_{1\text{cm}}^{1\%}$  328  $m\mu$  = 1800 approximately. Preliminary biological tests on the crystalline esters have been carried out by Dr Katharine Coward at the laboratories of the Pharmaceutical Society of Great Britain and by Dr S. W. F. Underhill in the physiological laboratories of the British Drug Houses Ltd. Both substances were found to be highly active and to have similar potencies when these were calculated on the basis of vitamin A content.

The acetate, diphenylacetate, 4-nitrobenzoate and stearate of vitamin A have also been prepared but have not as yet been obtained crystalline.

It was found that distilled vitamin A alcohol having  $E_{1\text{cm}}^{1\%} 328\text{ m}\mu = 1600^1$  could be crystallized from methyl alcohol at low temperatures. The product obtained in this way had properties identical with those of crystalline vitamin A which had been prepared by the method of Holmes and Corbet [1937]. When working with our raw materials, distillation, followed by direct crystallization, proved to be the less tedious procedure. Two batches prepared by the latter method had  $E_{1\text{cm}}^{1\%} 328\text{ m}\mu = 1800$  in ethyl alcohol and Carr-Price values 92,000 and 94,000.

The stability of the synthetic esters is of importance in connexion with the possibility of their use as biological standards. All the highly purified preparations of vitamin A and its esters which are described in this paper appear to deteriorate on storage in the absence of oxygen. The progress of this change is marked by a decrease in  $E_{1\text{cm}}^{1\%} 328\text{ m}\mu$  and in Carr-Price value and the rate at which it takes place varies considerably; the crystalline 2-naphthoate and anthraquinone-2-carboxylate were found to be the most stable preparations of high potency, although the specimens of which portions were used for the biological tests deteriorated slightly during three months' storage *in vacuo*: solutions of these esters in arachis oil, however, appear to be quite stable. Crystalline vitamin A alcohol (on the other hand) appears to be more labile: Holmes and Corbet observed that the ultraviolet absorption of solutions of the crystals decreased very rapidly and a similar falling off was observed by us with a crystalline specimen during storage *in vacuo* at a temperature below its melting point.

## EXPERIMENTAL

### *The purification of vitamin A by molecular distillation*

Concentrates of vitamin A, substantially free from sterols, were prepared from fish liver oils in the usual manner. These were further purified by repeated fractional molecular distillation in a cyclic still of the type described by Hickman [1937]. In this apparatus, the distilland may be re-circulated through the still for any desired number of times. The rate of circulation may be varied at will, but a speed of about 6 ml. per min. was found to give the best results. Each passage of the distilland over the hot distillation surface at a temperature which may be predetermined constitutes a cycle. The distillation surface, consisting of a vertical metal cylinder, is placed co-axially within an air-cooled glass tube which acts as the condenser. The space between the hot surface and the condenser is about 1 cm. wide, and is evacuated to  $1\text{--}3\mu$  by a condensation pump with a high pumping speed. The distillate flows down the condenser into a receiver which may be changed without interrupting the distillation. In using this apparatus for the distillation of viscous vitamin A concentrates, the latter were diluted with "residue oil" which was prepared from cod liver oil by removal of material volatilizing at or below  $250^\circ$  in a molecular still. Many distillations

<sup>1</sup> Smith *et al.* [1938] and Gillam & El Ridi [1938] have shown that the ultraviolet absorption of vitamin A preparations varies with the solvent used for the determination, while Holmes & Corbet have reported that Darby noticed a rapid decrease in the extinction coefficient of solutions of the crystalline vitamin. It is on account of these observations that we give the following particulars of the determination of the extinction coefficients quoted in this paper. Pure *isopropyl* alcohol was used as solvent in all cases except for the measurements on crystalline A when ethyl alcohol was employed. The determinations were made within 20–30 min. of the preparation of the solution and the latter was subjected for about 4 min. to the action of the light from a spark between electrodes of tungsten steel.

and redistillations were carried out during the preparation of the highly purified vitamin used in the experiments described in this paper. The results of two distillations are given in Table I.

Table I. *Distillation of crude vitamin A concentrate*

Fraction	Temperature °C.	Pressure $\mu$	No. of cycles	Wt. g.	Carr-Price value	Recovery %
Distilland: Run 7. Vitamin A concentrate, Carr-Price value 40,000						80 g.
"Residue oil" ... ..					120 g.	
1	95	4-7	5	6.2	64,000	12
2	110	1-3	5	20.7	66,000	42
3	120	1-2	4	14.8	62,500	29
4	130	1-2	2	6.7	56,000	11
Total recovery						94
Run 20. Mixed fractions from previous distillations, Carr-Price value 53,750						77 g.
"Residue oil" ... ..					20 g.	
1	85	2-3	3	6.9	67,500	10
2	95	1-2	5	11.5	80,000	22
3	105	1-2	5	17.2	68,750	29
4	115	1-2	3	11.5	66,250	18
5	125	1-2	7	10.3	55,000	14
Total recovery						93

Fraction 2 (run 20) had  $E_{1\text{cm}}^{1\%}$  328  $m\mu$  - 1690 (in alcohol). The purest fraction which we have obtained by molecular distillation had  $E_{1\text{cm}}^{1\%}$  328  $m\mu$  - 1950.

#### *Crystallization of distilled vitamin A alcohol*

A solution of vitamin A (20.4 g., Carr-Price value 81,000) in methyl alcohol (250 ml.) was cooled in solid carbon dioxide, inoculated with crystals obtained from a previous preparation, and allowed to warm up until only a small amount of solid remained undissolved. The bottle containing the solution was then covered with cotton wool and placed in a tin surrounded with solid carbon dioxide. After two days' storage at about  $-75^\circ$ , the pale yellow crystals were collected on a sintered glass filter, cooled with solid carbon dioxide. The material was pressed on a porous plate and dried during 3 hr. in a high vacuum, at a temperature which was allowed to rise slowly from  $-8$  to  $4^\circ$ . Yield 9.2 g.

This material when allowed to warm up slowly in an evacuated tube, melts at about  $8^\circ$  to a liquid which evolves vapour which may prove to be solvent of crystallization and simultaneously becomes more viscous. When completely freed from solvent in this manner, the product had Carr-Price value 92,000 and  $E_{1\text{cm}}^{1\%}$  328  $m\mu$  = 1820 (in ethyl alcohol).

#### *Vitamin A stearate*

Vitamin A alcohol (17.4 g., Carr-Price value 80,000) in ether, was treated with ethereal solutions of recrystallized stearyl chloride (16.2 g.), and triethylamine (17.4 g.). The solutions of the latter reagents were added in parallel portions and the reaction was carried out at  $0^\circ$ , and in an atmosphere of nitrogen. The mixture stood overnight at room temperature and was filtered to remove triethylamine hydrochloride. The filtrate was washed with water, aqueous  $\text{NaHCO}_3$  and water, and the ether was evaporated in nitrogen. In order to remove any unchanged vitamin A alcohol, the residue remaining after evaporation of the ether was dissolved in light petroleum (125 ml.), and the solution was allowed to percolate



through a column of activated alumina (150 g.). This material adsorbs the free vitamin A but not the esters. The column was rinsed with light petroleum and the filtrate and rinsings were evaporated under diminished pressure. The residue (17.3 g., Carr-Price value 48,000) consisted of a pale yellow oil which solidified to a wax at about 17°. This material was dissolved in ether and the solution was cooled in solid carbon dioxide; the pale yellow solid ester was collected and dried at a low temperature. The Carr-Price value of the product fell from 45,000 to 30,000 in 48 hr., and the experiment was therefore not pursued further.

#### *Vitamin A diphenylacetate*

Vitamin A (14 g., Carr-Price value 78,000) in pyridine (25 ml.) was treated with a solution of diphenylacetyl chloride (10 g.) in benzene (40 ml.). The mixture was warmed on the water bath for 60 min., then cooled and diluted with ethyl acetate. The solution was washed, dried and evaporated. The residue was dissolved in light petroleum and the solution was extracted ten times with an equal volume of 95 % methyl alcohol in order to remove unchanged vitamin A. The ester (4.9 g.) which remained after evaporation of the light petroleum had Carr-Price value 37,500, but could not be crystallized.

#### *Vitamin A 4-nitrobenzoate*

This ester, which was prepared in the usual manner by interaction between vitamin A and 4-nitrobenzoyl chloride in pyridine, formed a red oil which was soluble in ethyl acetate, acetone and ether, sparingly soluble in light petroleum, and insoluble in methyl alcohol. It was not obtained crystalline.

#### *Vitamin A acetate*

This compound, which was first described by Karrer [1931], was prepared by the interaction of distilled vitamin A (Carr-Price value 50,000) and redistilled acetyl chloride in pyridine. Since vitamin A and its acetate distil at approximately equal rates at the same temperature, the crude product was partitioned between methyl alcohol and light petroleum, in order to remove any vitamin that might have escaped esterification. For this, a solution of the acetate in light petroleum was extracted eight times with an equal volume of 95 % methyl alcohol. The acetate is somewhat soluble in methyl alcohol, and the process involved considerable loss. The acetate which was recovered from the petroleum layer had a Carr-Price value 34,000. It was diluted with residue oil and distilled. The most potent fraction distilled at 100° and consisted of a pale yellow, mobile oil with Carr-Price value 55,000 and  $E_{1\text{cm}}^{1\%} = 328$   $m\mu = 860$ .

#### *Vitamin A anthraquinone-2-carboxylate*

For the preparation of this ester, and of the 2-naphthoate, the method described by Hamano was used. The details are given in view of the difference in our starting material.

Distilled vitamin A alcohol (6.1 g., Carr-Price value 80,000) in pyridine (20 ml.) was treated with a solution of recrystallized anthraquinone-2-carboxyl chloride (4.3 g., m.p. 150°) in warm benzene (40 ml.). The mixture was heated on the water bath for 30 min. in an atmosphere of nitrogen, and the greater part of the solvents was then removed in a stream of this gas. The residue was dissolved in warm ethyl acetate (200 ml.). The solution was filtered and washed with water, dilute acid, water and aqueous  $\text{NaHCO}_3$ . After filtration to remove suspended sodium anthraquinone-2-carboxylate, and a final wash with water, the solution was dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated under diminished pressure. The

residue was dissolved in a small volume of acetone from which the ester soon began to separate in the form of small yellow plates. After two further crystallizations from acetone, the product formed macroscopic plates or prisms which melted at  $126^{\circ}$  (corr.) after turning red at about  $123^{\circ}$ . Hamano gives  $124^{\circ}$  as the melting point of the yellow modification of this ester. The Carr-Price value was 47,000 and the coefficient of absorption  $E_{1\text{cm}}^{1\%} 328 \text{ m}\mu = 938$ . These figures correspond to Carr-Price value 85,000 and  $E_{1\text{cm}}^{1\%} 328 \text{ m}\mu = 1510^1$  for vitamin A alcohol. (Found C. 80.4, 80.7 %; H. 6.97, 6.81 %;  $\text{C}_{35}\text{H}_{38}\text{O}_4$  requires C. 80.8 %, H. 6.92 %.)

The anthraquinone-2-carboxylate has also been obtained in the red form described by Hamano. This form was found to be less stable and more soluble at room temperature than the yellow allotrope. Conversion of the red into the yellow allotrope may be effected by solution in hot acetone, followed, after cooling, by inoculation of the solution with a crystal of the appropriate modification. After several recrystallizations from ethyl acetate-alcohol, the red modification formed long flat prisms having blunt ends, m.p.  $118\text{--}120^{\circ}$ . The Carr-Price value was 47,000 and the coefficient of absorption  $E_{1\text{cm}}^{1\%} 328 \text{ m}\mu = 1090$ . These figures correspond to Carr-Price value 85,000 and  $E_{1\text{cm}}^{1\%} 328 \text{ m}\mu = 1790^1$  for vitamin A alcohol.

#### *Vitamin A-2-naphthoate*

Vitamin A alcohol (17.8 g., Carr-Price value 80,000) in pyridine (30 ml.) was treated in an atmosphere of nitrogen with 2-naphthoyl chloride (8.2 g.) in benzene (40 ml.). The mixture was heated during 30 min. on a water bath and the greater part of the solvents was then distilled off in a stream of nitrogen. The residue was taken up in ether. Further working as described for the anthraquinone-carboxylate gave a pale yellow syrup which was dissolved in acetone (ca. 30 ml.). The solution was cooled to about  $-75^{\circ}$  as described by Hamano; the ester could not be induced to crystallize after several days at this temperature, and the solution was therefore treated with methyl alcohol (ca. 10 ml.). Crystallization started at once and was completed by cooling to  $-75^{\circ}$ . The precipitate, which was partly crystalline and partly gummy, was collected and recrystallized from a small volume of acetone in which the ester is rather soluble. After four crystallizations from ethyl alcohol, the product weighed 950 mg. and melted at  $78^{\circ}$  (corr.). Hamano gives  $76^{\circ}$  as the melting point of vitamin A 2-naphthoate.

The ester crystallized from alcohol as large pale yellow plates, and from acetone as pale yellow elongated prisms. The specimen used for the biological test (Part II) had, immediately after its preparation: Carr-Price value 50,000 and  $E_{1\text{cm}}^{1\%} 328 \text{ m}\mu = 1180$  corresponding to Carr-Price value 76,900 and  $E_{1\text{cm}}^{1\%} 328 \text{ m}\mu = 1790^2$  for the vitamin A alcohol. (Found: C, 83.9, 83.7 %; H, 8.06, 8.18 %.  $\text{C}_{31}\text{H}_{36}\text{O}_2$  requires C, 84.5 %; H, 8.18 %.)

Another specimen had  $E_{1\text{cm}}^{1\%} 328 \text{ m}\mu = 1190$  and Carr-Price value 62,500. These values correspond to  $E_{1\text{cm}}^{1\%} 328 \text{ m}\mu = 1800^2$  and Carr-Price value 96,000 for vitamin A.

#### *The effect of storage on the properties of the crystalline esters*

Samples of the batches of the anthraquinone-2-carboxylate and 2-naphthoate which were used for animal assays were stored in evacuated amber tubes. After about 3 months the esters were unaltered in appearance and had undergone no

<sup>1</sup> These values have been corrected for the absorption due to anthraquinone-2-carboxylic acid; ethyl anthraquinone-2-carboxylate was found to have  $\epsilon_{328} = 5600$ .

<sup>2</sup> These values have been corrected for the ultraviolet absorption of 2-naphthoic acid for which we found  $\epsilon_{328} = 820$ .

marked change in melting point. Further examination, however, disclosed that the ultraviolet absorption of both esters had decreased by about 20 % and that whereas the Carr-Price value of the naphthoate was practically unchanged, that of the anthraquinone carboxylate had decreased by about 15 %. This behaviour contrasted sharply with the apparent stability of the solutions of these esters in arachis oil, which were used for the biological tests. The effects of storage *in vacuo* are summarized in Table II. Exposure of the crystals to the air brought about rapid decomposition.

Table II

Ester	...	Anthraquinone-2-carboxylate	2-Naphthoate
Date	...	5. iii. 38	13. vi. 38
$E_{1\text{ cm}}^{1\%}$ 328 m $\mu$		938	750
Carr-Price value		47,000	40,000
M.P. (corr.)		126° after turning red at 123°	127° after turning red at 121°

17. iii. 38	13. iii. 38
1180	980
50,000	52,000
78°	77-79°

## PART II

Biological tests were carried out on the yellow modification of vitamin A anthraquinone-2-carboxylate and on vitamin A 2-naphthoate. Both esters were dissolved in arachis oil containing 0.01 % quinol, the anthraquinone-2-carboxylate at a concentration of 0.15 % (solution P. 87) and the 2-naphthoate at 0.2 % (solution P. 90). The solutions were kept under nitrogen in the refrigerator, dilutions being prepared at regular intervals for administration to the animals: the diluted solutions were also kept under the same conditions except when required for feeding. The coefficients of absorption and the Carr-Price blue values of the two solutions (P. 87 and P. 90) were determined at intervals during the course of the experiments (Table III): the results show that no deterioration occurred during this period. (Later determinations of the blue values have shown that the solutions have remained stable for 7 months.)

The biological tests were carried out by us independently, employing the technique customary in each laboratory.

Table III

P. 87			P. 90		
Date	$E_{1\text{ cm}}^{1\%}$ 328 m $\mu$	Carr-Price blue value	Date	$E_{1\text{ cm}}^{1\%}$ 328 m $\mu$	Carr-Price blue value
16. iii. 38	—	64	22. iii. 38	2.14	100
17. iii. 38	1.42	64	28. iii. 38	2.15	100
21. iii. 38	1.43	64	11. iv. 38	2.14	96
11. iv. 38	1.51	60	26. iv. 38	2.10	96
26. iv. 38	1.55	60	14. vi. 38	2.03	100
14. vi. 38	1.55	60			

## I. Determination of biological potency by S. W. F. U.

For the feeding experiments the solutions of the esters were further diluted with arachis oil containing quinol. P. 87 was diluted 1 : 6 and 1 : 12 and P. 90 1 : 10 and 1 : 20, that is, in both cases to make solutions with Carr-Price blue values of 10 and 5 respectively. Solutions containing 200 and 100 units per g. were similarly prepared from the solution of the international standard  $\beta$ -carotene. The dilutions were checked by determination of the blue value.

The biological tests were carried out on young rats which were given our vitamin A-free diet [Culhane, 1933] when they had reached a weight of about 40 g. each. When the weights became stationary the animals were divided into groups, those from different litters being distributed as evenly as possible amongst the different groups. All the groups received the dose of 0.02 g. of oil twice a week given by calibrated pipette directly into the animals' mouths. Three groups of each sex were given doses of the two esters and of the standard respectively. Another three groups of each sex were given a dose of ester or standard once a week only, alternately with a dose of arachis oil. The seventh group (negative controls) were given only doses of arachis oil twice a week.

Two experiments were carried out: in the first 78 animals were employed—51 bucks and 27 does; of these 8 bucks and 3 does were kept as negative controls. The doses were 0.02 g. of the solutions of the esters diluted 1:6 (P. 87) and 1:10 (P. 90) and four units of standard, given once and twice weekly. In the second experiment 57 animals were used—26 bucks and 36 does; 3 bucks and 4 does were kept as negative controls. The doses of the esters were 0.02 g. of the dilutions 1:12 (P. 87) and 1:20 (P. 90) with two units of standard, given once and twice weekly.

The average growth rate of the different groups in g. per week was calculated after the supplements had been given for three weeks. The results are given in Table IV.

Table IV

Exp. no.	Weekly dose	Dilution employed	♂		♀	
			No. of rats	Growth in g. per week	No. of rats	Growth in g. per week
Vitamin A anthraquinone-2-carboxylate: P. 87						
1	0.04 g.	1 : 6	4	9.8	5	7.5
	0.02 g.	1 : 6	4	7.0	6	5.0
2	0.04 g.	1 : 12	2	5.5	4	5.4
	0.02 g.	1 : 12	3	4.4	5	1.4
Vitamin A 2-naphthoate: P. 90						
1	0.04 g.	1 : 10	4	10.9	6	7.9
	0.02 g.	1 : 10	7	9.1	4	5.6
2	0.04 g.	1 : 20	3	4.3	4	5.3
	0.02 g.	1 : 20	4	2.3	4	1.8
International standard						
1	8 units		8	8.8	7	6.0
	4 "		6	7.6	6	3.2
2	4 "		6	1.4	5	1.7
	2 "		3	-0.7	7	-1.5

Since standard dose-response curves for bucks and does are not in use in this laboratory, curves relating the increase in weight to the logarithm of the dose were first calculated for each solution P. 87 and P. 90 and for the international standard. The average slope of the three curves was next obtained, making allowance for the number of animals used, and a curve with this slope adjusted (mathematically) to the midpoint of each of the individual curves, so that the ratios of the doses of P. 87 and P. 90 to the dose of standard could be found. From these figures the potency of each solution was calculated, the results given by the bucks and does being averaged, with weighting for the number of animals used. The mean potencies are given in Table V.

Table V. *Potency of solutions P. 87 and P. 90 in units per g.*

Exp. no.	P. 87	P. 90
1	1580	3830
2	4250	4880
Mean	2740	4280
Standard error	+35 % -26 %	+36 % -27 %

In the first experiment the average growth rate was also determined for a period of 5 weeks: the potencies of the solutions calculated from these figures are not significantly different from those given in Table V.

The potencies of the esters and of vitamin A alcohol were calculated from the mean potencies of the solutions P. 87 and P. 90. The potency of the anthraquinone-2-carboxylate is 1,827,000 units per g. and of the 2-naphthoate 2,140,000 units per g. The figures for the potency of vitamin A alcohol are, respectively, 3,320,000 and 3,293,000 units per g. Since  $\beta$ -carotene contains, by definition, 1,667,000 units of vitamin A activity per g., vitamin A is, weight for weight, twice as potent as  $\beta$ -carotene.

## II. *Determination of biological potency by K. H. C.*

The vitamin A potency of the two preparations was determined by the increase in weight brought about by giving doses to rats which had been fed on a vitamin A-free diet until they had become steady in weight. The details of the method have been fully described elsewhere [Coward, 1938].

The amount of vitamin A in P. 87 was theoretically 0.0825 % (mol. wt. vitamin A anthraquinone-2-carboxylate 520: mol. wt. vitamin A alcohol 286). The dose of solution tested was 0.5 mg. (diluted with arachis oil 1:20) per day in comparison with one unit of the international standard in 10 mg. arachis oil.

The amount of vitamin A in P. 90 was theoretically 0.13 % (mol. wt. vitamin A 2-naphthoate 440). The dose of solution tested was 0.33 mg. (diluted with arachis oil 1:30) per day in comparison with 0.5 mg. of the first solution and one unit of the international standard in 10 mg. arachis oil.

The results were calculated in the way in general use in the laboratories of the Pharmaceutical Society. They are summarized in Table VI.

Table VI

	Dose	No. of rats		Increase in wt.		Corresponding abscissae on curves of response		Weighted mean of abscissae
		♂	♀	♂ g.	♀ g.	♂	♀	
P. 87	0.5 mg.	5	9	13.0	15.3	1.72	2.48	2.21
P. 90	0.33 mg.	5	7	26.6	13.4	4.94	1.94	3.19
Vit. A	1 I.U.	4	10	22.5	10.0	3.60	1.25	1.92

$$\text{P. 87.} \quad \frac{\text{The vitamin A content of 0.5 mg. P. 87}}{\text{1 I.U. of vitamin A}} = \frac{2.21}{1.92} = 1.15.$$

Therefore 0.5 mg. P. 87 contains 1.15 units vitamin A and the solution contains 2300 I.U. of vitamin A per g.

$$\text{P. 90.} \quad \frac{\text{The vitamin A content of 0.33 mg. P. 90}}{\text{1 I.U. of vitamin A}} = \frac{3.19}{1.92} = 1.66.$$

Therefore, 0.33 mg. P. 90 contains 1.66 units vitamin A and the solution contains 5000 I.U. of vitamin A per g.

The potency of vitamin A according to the first test:

$$\text{Dose of vitamin A given was } 0.0005 \times \frac{0.0825}{100} \text{ g.} = 0.0000004125 \text{ g.} \\ = 0.4125 \mu\text{g.}$$

$$\frac{0.4125 \mu\text{g. vitamin A}}{0.6 \mu\text{g. } \beta\text{-carotene}} = \frac{2.21}{1.92}$$

Therefore vitamin A is 1.67 times as potent as  $\beta$ -carotene.

The potency of vitamin A according to the second test:

$$\text{Dose of vitamin A given was } 0.00033 \times \frac{0.13}{100} \text{ g.} = 0.000000429 \text{ g.} \\ = 0.429 \mu\text{g.}$$

$$\text{Therefore } \frac{0.429 \mu\text{g. vitamin A}}{0.6 \mu\text{g. } \beta\text{-carotene}} = \frac{3.19}{1.92}$$

Therefore vitamin A is 2.32 times as potent as  $\beta$ -carotene.

The average of these two figures gives the potency of vitamin A as twice that of  $\beta$ -carotene, weight for weight.

#### DISCUSSION

We consider that our results, which were obtained independently in different laboratories, show reasonably good agreement: they are summarized in Table VII.

Table VII

	P. 87 Vitamin A anthraquinone 2-carboxylate 0.15 %	P. 90 Vitamin A 2-naphthoate 0.2 %
Concentration of solution in arachis oil		
$E_{1\text{ cm}}^{1\%}$ 328 m $\mu$	1.49	2.11
Carr-Price blue value	60	100
Potency: Units per g. S. W. F. U. 1	1580	3830
S. W. F. U. 2	4250	4880
K. H. C.	2300	5000
Mean	2625	4450
"Conversion factor" (corr.)	1920	2150

The potencies of the esters and of vitamin A alcohol calculated from these figures are:

	Units per g.		Units per g.
Vitamin A anthraquinone-2-carboxylate	1,750,000	Vitamin A alcohol	3,181,000
Vitamin A 2-naphthoate	2,225,000	Vitamin A alcohol	3,424,000

In the case of each ester the calculated potency of the vitamin A alcohol is approximately twice that of  $\beta$ -carotene.

For some years it has been assumed that the molecule of  $\beta$ -carotene breaks up in the animal body and by the addition of an OH group and an H atom to each of the broken ends, two new molecules are formed, each of which is a molecule of vitamin A. Thus 536 g. of  $\beta$ -carotene would give 572 g. of vitamin A and vitamin A and  $\beta$ -carotene would have very nearly equal activities weight for weight. But the two samples of vitamin A examined biologically have been shown to have 1.91 and 2.05 times respectively the vitamin A potency of  $\beta$ -carotene. (The difference between these figures is not significant.) This is in agreement with the results of Holmes & Corbet [1937] who found the potency of their vitamin A to be 3,000,000 I.U. per g. These results indicate either that (a)  $\beta$ -carotene is not converted quantitatively into vitamin A in the animal body or that (b) the molecule

of  $\beta$ -carotene gives rise to only one molecule of vitamin A, the other part of the molecule having no activity either when it was part of the carotene molecule or when it was separated from it. The latter possibility seems to be the more probable one since otherwise it is unlikely that the several tests should all have indicated that vitamin A has double the potency of  $\beta$ -carotene, weight for weight. Also it might be expected that, if  $\beta$ -carotene is not converted quantitatively into vitamin A in the body, the variation in different rats' powers of conversion would result in a greater variation in response to a given dose of carotene than that in response to a dose of any other form of vitamin A. Coward [1936] has shown that the standard deviation of the response of rats which had been given vitamin A in the form of carotene in vegetables was not greater than, and was apparently a little less than, that of rats given true vitamin A in cod liver oil, butters and vitaminized margarine (Table VIII).

Table VIII

	No. of rats from which the calculation was made		Standard deviation	
	♂	♀	♂	♀
1. Tests on a cod liver oil, Z	510	645	11.58	9.51
2. Tests on (a) butters and (b) margarines	257 281	315 371	12.75 11.45	9.72 10.03
1 and 2 together	1048	1331	11.85	9.71
*Tests on $\beta$ -carotene, the international standard	207	243	8.95	7.84

\* Not before published.

A calculation has since been made of the standard deviation of the response of rats to doses of the international standard (i.e.  $\beta$ -carotene in oil) (Table VIII). It was found to be less than that of rats given doses of vitamin A in any other form and the difference was almost certainly significant.

$\frac{\sigma_1 - \sigma_2}{\sqrt{(\epsilon_1^2 + \epsilon_2^2)}}$ , in which  $\sigma$  is the standard deviation of a single observation of increase in weight, and  $\epsilon = \frac{\sigma}{\sqrt{\{2(n \cdot m)\}}}$ ,  $n$  being the number of rats and  $m$  the number of groups of rats, was 5.06 for the male rats and 4.23 for the female rats. Thus the variation in response to doses of  $\beta$ -carotene in oil is less than the variation in response to doses of true vitamin A in oil, and therefore the  $\beta$ -carotene is probably not incompletely converted into vitamin A in the animal body.

It must be concluded, therefore, that the molecule of  $\beta$ -carotene has only one active group and that, on breaking down in the animal body, only one molecule of vitamin A is formed from one molecule of  $\beta$ -carotene, the other part of the carotene molecule presumably being converted into some substance which is without any vitamin A activity.

The fact that the calculated potency of vitamin A is the same in the case of each ester shows that the vitamin in each is assimilated by the rat to the same extent: it is of interest that esters which are not natural to the body can be apparently completely utilized. Our results taken in conjunction with those of Holmes & Corbet afford strong evidence that the rat utilizes indifferently and probably completely vitamin A and its natural esters. Utilization of the synthetic esters presumably depends upon their hydrolysis in the body: it will be of interest if a non-hydrolysable and, therefore, inactive ester is prepared in the future.

It has been stated [Wilkinson, 1938] that the curve of response for vitamin A is different from that for carotene. This can be tested for these experiments by comparing the increases in weight with the pairs of doses of esters and international standard in Table IV, since the slope of the curve for each pair of doses is formed by dividing the difference between the increases in weight by the difference between the logarithms of the doses given, which in these experiments is always 0.301. For the male rats, the differences between the increases in weight from pairs of doses of the esters were 2.8, 1.1, 1.8 and 2.0 and for the female rats, 2.5, 4.0, 2.3, 3.5: the corresponding figures from pairs of doses of the standard were for the male rats 1.2 and 2.1, and for the female rats 2.8 and 3.2. It is obvious, without further calculation, that the slopes of the curves of response for vitamin A are, under the conditions of this experiment at least, not different from those for  $\beta$ -carotene. Since pairs of doses were not given in the experiment recorded in Table VI, it is not possible to test these figures in the same way.

We have also calculated from our figures the value of the "conversion factor" for converting the value of the extinction coefficient of vitamin A into biological units per g. For this purpose it is necessary to correct the observed  $E_{1\text{cm}}^{1\%}$  328  $m\mu$  values (Table III) for the absorption due to the acid fraction of the ester molecules. The corrections are for P. 87, 0.12, and for P. 90, 0.04, and the observed values must be reduced by these amounts. The value of the conversion factor for the vitamin A anthraquinone-2-carboxylate solution is then 1920 and for the solution of the vitamin A 2-naphthoate, 2150: the difference between these figures is not significant. The factor accepted for the B.P. addendum 1936 is 1600, but our results are within the upper range of the figures from which the average of 1600 was obtained. They suggest, however, that the true value for the "conversion factor" for pure vitamin A is about 2000. Two explanations may be offered for the variations from this figure which have frequently been observed; either the solution examined has contained material which absorbs at wave lengths in the region of 328  $m\mu$ , but is not vitamin A or vitamin A in the form in which it is usually found, or the absorption of the vitamin from the intestine, or its utilization in the body of the rat varies according to its source, or according to the diet on which the animals are maintained. Our own results incline us to favour the former explanation although they do not exclude the latter.

## SUMMARY

### Part I

1. Vitamin A has been obtained in a high state of purity by distillation in the Hickman cyclic still.
2. The distilled vitamin has been crystallized directly from methyl alcohol at a low temperature.
3. The stearate, 4-nitrobenzoate, diphenylacetate and acetate have been prepared from highly purified vitamin A, but have not been obtained crystalline.
4. The crystalline anthraquinone-2-carboxylate and 2-naphthoate of vitamin A have been prepared from material purified by molecular distillation. These esters were found to resemble the anthraquinone-2-carboxylate and 2-naphthoate prepared by Hamano from undistilled concentrates.

### Part II

1. The biological potencies of two esters of vitamin A have been determined in terms of the international standard  $\beta$ -carotene in simultaneous tests.
2. Vitamin A anthraquinone-2-carboxylate was found to contain 1,750,000 units per g. and vitamin A 2-naphthoate 2,225,000 units per g.



3. The potency of vitamin A alcohol is therefore 3,181,000 I.U. per g. according to the tests on the first ester and 3,424,000 I.U. per g. according to the tests on the second ester.

4. Since, by definition,  $\beta$ -carotene contains 1,670,000 I.U. of vitamin A activity per g., vitamin A itself is twice as active as  $\beta$ -carotene, weight for weight.

5. The factor for converting the coefficient of absorption at 328  $m\mu$  into biological units per g. ("conversion factor") was found to be about 2000.

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# LXXIII. THE WATER-SOLUBLE B-VITAMINS

## XIII. ALLOXAZINE-ADENINE-DINUCLEOTIDE, ADENYLIC ACID, NICOTINAMIDE AND PIMELIC ACID IN THE NUTRITION OF THE RAT

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RECENT investigations carried out in this laboratory have shown that the vitamin B requirements of the rat are satisfied only when the animals receive aneurin, riboflavin and three additional heat-stable factors belonging to what is known as the vitamin B<sub>2</sub> complex [Edgar *et al.* 1938, 3]. One of these factors, named yeast eluate factor, is contained in eluates prepared from fuller's earth adsorbates of autoclaved aqueous yeast extracts and has been identified [El-Sadr *et al.* 1939] with Lepkovsky's factor 1 [Lepkovsky, 1938] and György's vitamin B<sub>6</sub> [György, 1938, 1; Keresztesy & Stevens, 1938; Kuhn & Wendt, 1938]. A second factor, which so far has not been isolated in a pure state, is present in fuller's earth filtrates of autoclaved aqueous yeast extracts and in certain preparations from liver, and has been named yeast or liver filtrate factor according to the source. The existence of a third factor is indicated by the fact that rats receiving crude extracts of yeast or liver, as source of the vitamin B complex, increased in body-weight more rapidly than did rats receiving adequate amounts of aneurin, riboflavin, yeast eluate factor and yeast filtrate factor. Concentrates of this third factor have now been prepared by a method which will be described in a later paper.

A description is here given of experiments in which we studied the possibility of replacing for the nutrition of the rat certain of the above-recognized members of the vitamin B<sub>2</sub> complex by substances which, in other types of investigations, have been found to be biologically important. We have examined (A) alloxazine-adenine-dinucleotide, (B) a combination of yeast adenylic acid and nicotinamide and (C) pimelic acid.

A. *Alloxazine-adenine-dinucleotide.* Warburg & Christian [1938, 1] have recently isolated from liver and yeast a riboflavin-containing dinucleotide (alloxazine-adenine-dinucleotide), which is the coenzyme of *d*-amino-acid dehydrogenase, of xanthine dehydrogenase and of other enzymes [Warburg & Christian, 1938, 2; Ball, 1938; Haas, 1938]. It was to be expected that this important coenzyme would replace riboflavin in the diet of the rat, and it seemed possible that it might also possess the vitamin activity of some other member of the vitamin B complex. We have been able to investigate this problem through the kindness of Prof. Warburg, who put an adequate amount of the coenzyme at our disposal.

B. *Combination of yeast adenylic acid and nicotinamide.* Nicotinic acid or nicotinamide has been shown to be an essential nutrient for man [Fouts *et al.* 1937; Smith *et al.* 1937; Spies *et al.* 1938], the dog [Elvehjem *et al.* 1937], the pig [Chick *et al.* 1938, 1] and the monkey [Harris, 1938]. Frost & Elvehjem [1937]

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reported that nicotinamide and adenylic acid from yeast or heart had growth-promoting properties when administered, either separately or simultaneously, to rats receiving a basal diet which contained 12 % of white corn. Later, however, Oleson *et al.* [1939] found no growth-stimulating action of nicotinic acid when given to rats on an entirely "synthetic" diet containing riboflavin. Euler & Malmberg [1936] stated that adenylic acid had no effect on the growth-rate of rats receiving a diet containing riboflavin, but that nicotinamide prolonged the life of rats receiving riboflavin and a yeast fuller's earth filtrate. More recently cozymase was found to have a growth-stimulating effect when administered to rats receiving riboflavin and a fuller's earth filtrate of an autoclaved acidulated yeast extract [Euler *et al.* 1938].

In this laboratory we were unable to demonstrate that nicotinamide, nicotinic acid or codehydrogenase II were dietary essentials for the rat [Macrae & Edgar, 1937]. Cook *et al.* [1937] also failed to find that nicotinic acid alone or in combination with adenylic acid had any vitamin-like action in the rat. Chick *et al.* [1938, 2] noted no increased growth when rats fed on a maize diet received nicotinamide, and Chick (unpublished experiments) was also unable to demonstrate that nicotinamide had a growth-stimulating effect when fed to rats receiving a purified synthetic basal diet containing sucrose as source of carbohydrate. Helmer & Fouts [1938], on the other hand, reported that nicotinic acid did increase the growth-rate of rats receiving a maize diet containing riboflavin and liver filtrate fraction: György [1938, 2] has attributed an anaemia of rats, termed panmyelophthisis, to deficiency of nicotinic acid in the diet. Dann & Subbarow [1938] stated that nicotinic acid given to rats receiving a diet containing riboflavin had no growth-promoting action and did not prevent rat dermatitis, i.e. could not replace vitamin B<sub>6</sub>.

In the present paper we report an extension of our former experiments [Macrae & Edgar, 1937] by investigating a combination of yeast adenylic acid and nicotinamide for vitamin activity.

C. *Pimelic acid*. Since the chemical behaviour of yeast filtrate factor proved it to be acidic in character [Edgar & Macrae, 1937, 2], it was thought possible that it might be identical with pimelic acid, a growth factor for *C. diphtheriae* [Mueller, 1937]. We have therefore tested pimelic acid for filtrate factor activity.

#### METHODS AND RESULTS

Growth of young rats was the criterion of vitamin activity employed; the basal diet and general experimental procedure have been described in previous papers from this laboratory [Chick *et al.* 1935; Edgar *et al.* 1937; 1938, 1]. All animals received 10 µg. aneurin and 0.08 ml. cod liver oil daily from the time of weaning until a body-weight of 100 g. was attained; the daily supplements were then increased to 15 µg. and 0.1 ml. respectively.

Synthetic aneurin and riboflavin were used throughout and the purified preparations of yeast and liver eluate factor and of yeast filtrate factor were those described in earlier papers [Edgar *et al.* 1937; Edgar & Macrae, 1937, 1; Edgar *et al.* 1938, 1, 2].

##### A. Investigation of the vitamin B<sub>2</sub> activity of alloxazine-adenine-dinucleotide

The sample of alloxazine-adenine-dinucleotide which we received from Prof. Warburg was a preparation of the monobarium salt obtained from yeast; it contained about 5 % of impurities. The material was fed to rats as the sodium salt, prepared by the addition of Na<sub>2</sub>SO<sub>4</sub> to a solution of the barium salt.

Four separate experiments were carried out. Groups of rats received one of the following combinations of vitamin B<sub>2</sub> factors: (a) yeast eluate fraction and yeast filtrate fraction, (b) riboflavin and yeast eluate fraction, (c) riboflavin and yeast filtrate fraction, (d) riboflavin, yeast eluate and yeast filtrate fractions. The effect of the addition to the diets of alloxazine-adenine-dinucleotide on the growth-rate of the rats in these groups was observed.

(a) *Addition of alloxazine-adenine-dinucleotide to the diet containing yeast eluate factor and yeast filtrate factor (Table I A).*

These experiments were carried out to determine how efficiently the coenzyme replaced riboflavin in the diet of the rat.

Previous experiments had shown that rats receiving diets containing yeast filtrate and yeast eluate fractions, but no riboflavin, failed to grow, but gained rapidly in weight when riboflavin was subsequently added to the diet [cf. Edgar & Macrae, 1937, 1 (Fig. 2)]. The following experiment carried out in a similar manner with alloxazine-adenine-dinucleotide instead of riboflavin, indicated that this coenzyme could replace riboflavin in the diet of the rat. Two rats which received only yeast eluate and filtrate fractions as sources of the vitamin B<sub>2</sub> complex increased in weight at an average rate of 2.1 g. weekly for 3 weeks: after receiving an additional daily supplement of an amount of alloxazine-adenine-dinucleotide equivalent to 20  $\mu$ g. riboflavin, the average weekly gain in weight during two subsequent weeks was 15.0 g.

A quantitative comparison of the vitamin activities of riboflavin and the coenzyme was, however, thought desirable.

The curve of response of body-weight increase to riboflavin dosage in animals receiving yeast eluate and yeast filtrate fractions as sources of the supplementary factors of the vitamin B<sub>2</sub> complex is a logarithmic one, similar to that obtained when yeast filtrate fraction is the only supplement given [Edgar *et al.* 1937]. We found optimum growth only when 40  $\mu$ g. riboflavin daily were administered to each rat: the growth-rate, however, was most sensitive to small changes in the riboflavin content of the diet when the animals received only about one-quarter of the optimal amount.

Accordingly, the relative vitamin potencies of riboflavin and alloxazine-adenine-dinucleotide were determined by comparing the increase in body-weight of rats receiving only 6 or 12  $\mu$ g. of riboflavin with that of animals receiving amounts of coenzyme equivalent to 6 or 12  $\mu$ g. of riboflavin.

Animals from two litters of eight rats each received at weaning the basal diet supplemented by aneurin and cod liver oil. For the first few days the animals increased slightly in body-weight but this increase ceased after 7–10 days. Four of the animals from one of the litters each now received daily doses of yeast filtrate fraction purified by amyl alcohol extraction (=2 g. dry yeast), yeast eluate fraction (=2 g. dry yeast) and an amount of alloxazine-adenine-dinucleotide equivalent to 6  $\mu$ g. riboflavin. Their four litter-mates received the same doses of yeast filtrate and eluate fractions but, instead of the coenzyme, 6  $\mu$ g. of riboflavin daily. The rats of the second litter were similarly treated, but received double the amounts of coenzyme and riboflavin. The growth-rate of all rats was observed for 4 weeks after the supplements were given.

All rats showed immediate growth responses when the supplements were added (Table I A). The four animals receiving 6  $\mu$ g. of riboflavin daily maintained an average growth-rate of 8.3 g. weekly for the 4-week period, and those receiving an equivalent amount of coenzyme gained an average of 7.4 g. weekly. Similarly,

Table I. *Effect of alloxazine-adenine-dinucleotide on the growth-rate of rats receiving aneurin and various other factors of the vitamin B complex*

No. of rats	Daily supplements of the vitamin B complex				Av. weekly wt. increases of group during 4 weeks after giving supplements g.	Mean weekly wt increase g.
	Yeast eluate factor = 2 g. dry yeast	Yeast filtrate factor = 2 g. dry yeast	6 $\mu$ g. riboflavin			
4	"	"	Alloxazine-adenine-dinucleotide = 6 $\mu$ g. riboflavin	11.2, 6, 8.2, 8	8.3	
4	"	"	12 $\mu$ g. riboflavin	9.5, 4, 8.5, 7.8	7.4	
4	"	"	Alloxazine-adenine-dinucleotide = 12 $\mu$ g. riboflavin	19, 14, 14.5, 12.5	15.0	
4	"	"		18.5, 15.2, 12.2, 9.8	13.9	
No. of rats	B				Av. weekly wt. increases during 2 weeks after giving S g.	Mean weekly wt. increase g.
	Daily supplements during preliminary period of 2 weeks	Av. weekly wt. increases during preliminary period g.	Mean weekly wt. increase g.	Additional supplement added daily (=S)		
4	50 $\mu$ g. riboflavin + yeast eluate factor = 2 g. dry yeast	18.5, 9.8	—	Alloxazine-adenine-dinucleotide = 20 $\mu$ g. riboflavin	9.0, 5.5	7.2
2	"	21.5, 8.5	—	Alloxazine-adenine-dinucleotide = 40 $\mu$ g. riboflavin	11.5, 2.5	7.0
4	"	20.2, 10	—	None	9.5, 5	7.2
1	"	20, 12	—	Yeast filtrate factor = 1 g. dry yeast	17, 18	17.5
No. of rats	C				Av. weekly wt. increases of group during 4 weeks after giving supplement g.	Mean weekly wt. increase g.
	Daily supplements of the vitamin B <sub>2</sub> complex	Alloxazine-adenine-dinucleotide given daily	Equivalent of 24 $\mu$ g. riboflavin			
3	50 $\mu$ g. riboflavin + yeast filtrate factor = 1 g. dry yeast	16, 13.3	14.6	Alloxazine-adenine-dinucleotide = 24 $\mu$ g. riboflavin	13, 13.7	13.3
2	"	14.5, 12.5	13.5	Liver eluate factor = 12 g. fresh liver	23.5, 22.5	23
No. of rats	D				Av. weekly wt. increases of group during 4 weeks after giving supplement g.	Mean weekly wt. increase g.
	Daily supplements of the vitamin B <sub>2</sub> complex	Alloxazine-adenine-dinucleotide given daily	Equivalent of 24 $\mu$ g. riboflavin			
4	50 $\mu$ g. riboflavin + eluate factor = 2 g. dry yeast + yeast filtrate factor = 2 g. dry yeast				19.8, 20.5, 20.5, 21.2	20.5
3	"		None		22, 19.3, 21, 22.7	21.2

the two groups of animals receiving daily 12  $\mu$ g. of riboflavin or alloxazine-adenine-dinucleotide equivalent to that amount of riboflavin increased in body-weight at approximately the same rate, the average weekly weight increases being 15 g. and 13.9 g. respectively. It is therefore concluded that, when administered orally, alloxazine-adenine-dinucleotide replaces riboflavin in the diet of the rat, and that equimolecular amounts of the coenzyme and riboflavin have the same vitamin potency.

*(b) Addition of alloxazine-adenine-dinucleotide to the diet containing riboflavin and yeast eluate factor (Table I B).*

Eleven rats received for 1 week from weaning the basal diet supplemented by aneurin and cod liver oil, and during a further preliminary period of 2 weeks each received daily, in addition, 50  $\mu$ g. riboflavin and yeast eluate fraction equivalent to 2 g. dry yeast. During the subsequent test period of 2 weeks, four of the animals received the additional supplement of an amount of alloxazine-adenine-dinucleotide equivalent to 20  $\mu$ g. riboflavin daily, two received an amount equivalent to 40  $\mu$ g. riboflavin, one serving as a positive control received an amount of yeast filtrate factor not purified by amyl alcohol extraction equivalent to 1 g. dry yeast, while four rats served as negative controls and received no further supplement. In this experiment the addition of alloxazine-adenine-nucleotide to the diet had no effect in restoring growth in the six rats receiving it (Table I B), the weekly weight increases being no greater than those of the negative controls. In the positive control rat, however, the growth-rate was restored.

*(c) Addition of alloxazine-adenine-dinucleotide to the diet containing riboflavin and yeast filtrate factor (Table I C).*

Five rats were maintained for 1 week from weaning on the basal diet supplemented by aneurin and cod liver oil, then for the further preliminary period of 2 weeks each received additional daily supplements of 50  $\mu$ g. riboflavin and of an amount of yeast filtrate factor not purified by amyl alcohol extraction equivalent to 1 g. dry yeast. During the subsequent test period of 2 weeks, daily supplements of the coenzyme equivalent to 24  $\mu$ g. riboflavin were given to three of the rats, and eluate fraction, equivalent to 12 g. fresh liver, to the other two animals which served as positive controls. The addition of the coenzyme caused no increase in the growth-rate, while the addition of the eluate fraction caused the usual marked increase (Table I C).

*(d) Addition of alloxazine-adenine-dinucleotide to the diet containing riboflavin yeast eluate factor and yeast filtrate factor (Table I D).*

Seven male litter-mate rats at weaning received the basal diet supplemented by aneurin and cod liver oil for 1 week. Four of the animals then each received daily 50  $\mu$ g. riboflavin, yeast eluate fraction equivalent to 2 g. dry yeast, yeast filtrate fraction purified by amyl alcohol extraction equivalent to 2 g. dry yeast, and alloxazine-adenine-dinucleotide equivalent to 24  $\mu$ g. riboflavin. Three animals which served as negative controls received all these fractions except the coenzyme. There was no significant difference in the rates of growth of the two groups (Table I D), the average weekly gains in weight during a 4-week period being 20.5 and 21.2 g. respectively.

The usual weekly weight increase of similar male rats receiving unfractionated extracts of yeast or liver as sources of the whole vitamin B<sub>2</sub> complex is more than 30 g. The amounts of riboflavin and yeast filtrate and eluate factors administered were more than enough to supply the animals' needs of those factors; this experiment proves, therefore, (a) that at least one factor of the vitamin B<sub>2</sub> complex,

in addition to those factors, is required by the rat and (b) that alloxazine-adenine-dinucleotide cannot replace this additional factor.

The above experiments indicate that the only vitamin activity possessed by alloxazine-adenine-dinucleotide is that which it has by virtue of the riboflavin it contains bound in its molecule, and that when it is administered orally its vitamin potency is identical with that of an equimolecular amount of riboflavin.

Warburg & Christian [1938, 2] suggest that alloxazine-adenine-dinucleotide is probably the form in which riboflavin functions in the animal body, but it is also possible that riboflavin phosphoric acid or free riboflavin or other unknown compounds of riboflavin may be concerned with processes in the living cell. These experiments show, however, that riboflavin and alloxazine-adenine-dinucleotide, when administered orally to the rat, are converted with equal efficiency into the compound or compounds which function in the animal's tissues.

*B. Investigation of the vitamin B<sub>2</sub> activity of a combination of yeast adenylic acid and nicotinamide*

The yeast adenylic acid was obtained from British Drug Houses, Ltd., and the nicotinamide from Messrs Hoffmann La Roche. These compounds were tested for vitamin activity by administration to rats receiving supplements of (a) riboflavin, (b) riboflavin and yeast eluate fraction and (c) riboflavin and yeast filtrate fraction.

*(a) Addition of yeast adenylic acid and nicotinamide to the diet containing riboflavin (Table II A).*

Six litter-mate rats received for 1 week from weaning the basal diet supplemented by aneurin and cod liver oil. Three animals which served as negative controls then each received a daily supplement of 50  $\mu$ g. riboflavin only, while the remaining three animals were each given daily 50  $\mu$ g. riboflavin, 2 mg. yeast adenylic acid and 2 mg. nicotinamide; the doses of the last two of these compounds after 1 week were changed to 1 mg. of each daily. The body-weights of the animals were observed for 6 weeks (Table II A). The average total weight increase during the 6 weeks subsequent to the animals receiving adenylic acid and nicotinamide was 25 g., while the corresponding average for the control rats was 33 g. This difference, however, cannot be regarded as significant and there was no apparent difference in the general condition of the rats of the two groups; all the animals were much undersized but were active. Two rats of the control group receiving only riboflavin developed the characteristic florid rat dermatitis [Copping, 1936] after 6 and 15 weeks respectively from the beginning of the experiment, while two of the animals receiving riboflavin, adenylic acid and nicotinamide developed the dermatitis after 8 and 15 weeks respectively. Addition of adenylic acid and nicotinamide therefore did not influence either the growth of the rats or the development of dermatitis.

*(b) Addition of yeast adenylic acid and nicotinamide to the diet containing riboflavin and yeast eluate factor (Table II B).*

Four litter-mate female rats received at weaning the basal diet supplemented by aneurin and cod liver oil and for a preliminary period of 2 weeks additional supplements were given of 50  $\mu$ g. riboflavin and yeast eluate fraction equivalent to 2 g. dry yeast. Three of the rats then each received for a further period of 3 weeks 2 mg. yeast adenylic acid and 2 mg. nicotinamide daily, while one animal received an amount of yeast filtrate fraction not purified by amyl alcohol extraction equivalent to 1 g. dry yeast. This last animal showed the usual growth

Table II. *Effect of a combination of yeast adenylic acid and nicotinamide on the growth-rate of rats receiving daily 10-15  $\mu$ g. aneurin, 50  $\mu$ g. riboflavin, and various other factors of the vitamin B<sub>2</sub> complex*

No. of rats	No. of rats	A		B		Av. wt. increase during week after giving N <sub>2</sub> g.
		Daily supplements	Av. weekly wt. increases of group during 6 weeks after dosing g.	Additional daily supplement added at end of preliminary period (N <sub>1</sub> )	Av. weekly increases during 2 or 3 weeks after giving N <sub>1</sub> g.	
	3	2 mg. adenylic acid + 2 mg. nicotinamide*	10.3, 4.7, 3.7, 2.3, 2.3, 1.7		Mean weekly wt. increase g.	Further supplement added (N <sub>2</sub> )
	3	None	15, 6.7, 5.0, 4.0, 1.0, 1.3			
	3	Yeast filtrate factor = 2 g. dry yeast	14.0, 9.3	2 mg. adenylic acid + 2 mg. nicotinamide	3, 6.3, 4	Yeast filtrate factor - 1 g. dry yeast
1	"	"	16.0, 7.0	Yeast filtrate factor - 1 g. dry yeast	13, 16, 16	—
3	Yeast filtrate factor = 1 g. dry yeast		20.3, 21, 13.7	(C. Exp. 1) 2 mg. adenylic acid - 2 mg. nicotinamide	15, 17, 13.7	Yeast eluate factor = 2 g. dry yeast
4	Yeast filtrate factor = 1 g. dry yeast		19.2, 15.2	(C. Exp. 2) 2 mg. adenylic acid + 2 mg. nicotinamide	14.2, 15.2, 11.8	—
2	"		18.5, 16.0	None	16, 16.5, 17	—
2	"		21.5, 15.0	Yeast eluate factor - 2 g. dry yeast	31.5, 28.5	—

\* After 1 week supplements of adenylic acid and nicotinamide reduced to 1 mg. of each daily.



response, but no increased growth-rate followed the administration to the test animals of adenylic acid and nicotinamide (Table IIB). At the end of the 3-week period addition of yeast filtrate fraction to the diet of these animals caused a striking increase in growth-rate, indicating that these amounts of adenylic acid and nicotinamide, although unable to stimulate growth, did not inhibit the growth of the animals when the missing nutrient was supplied. The increase in growth-rate caused by addition of filtrate factor, however, was not as great as that which follows administration of unfractionated yeast extracts to such animals; therefore, these compounds cannot replace that factor of the vitamin B<sub>2</sub> complex required in addition to riboflavin, eluate factor and filtrate factor.

(c) *Addition of yeast adenylic acid and nicotinamide to the diet containing riboflavin and yeast filtrate fraction (Table IIC).*

Three rats which had received the basal diet with aneurin and cod liver oil for 1 week from weaning were each given daily supplements of 50  $\mu$ g. riboflavin and an amount of yeast filtrate fraction not purified by amyl alcohol extraction equivalent to 1 g. dry yeast for a period of 3 weeks; 2 mg. yeast adenylic acid and 2 mg. nicotinamide were then given daily for a further period of 3 weeks, but there was no increase in the growth-rate; increased growth-rate, however, did occur when a daily ration of yeast eluate fraction equivalent to 2 g. dry yeast was added (Table IIC, Exp. 1).

In a second experiment eight litter-mate rats, treated as usual for the first week after weaning, each received daily 50  $\mu$ g. riboflavin and an amount of yeast filtrate fraction equivalent to 1 g. dry yeast for a period of 2 weeks. Four of the animals then each received the additional daily supplements of 2 mg. adenylic acid and 2 mg. nicotinamide for 3 weeks, two negative control animals received no additional supplement during that period and two positive control animals received yeast eluate fraction equivalent to 2 g. dry yeast daily. The animals receiving the adenylic acid and nicotinamide increased in body-weight at approximately the same rate as the negative controls while the growth-rates of the two positive control rats increased from an average of 18.2 g. weekly to 30 g. weekly on addition of the eluate factor (Table IIC, Exp. 2).

The results of the above experiments afford no evidence that yeast adenylic acid and nicotinamide administered simultaneously have any growth-promoting activity for rats deprived of the yeast eluate factor (vitamin B<sub>6</sub>) or yeast filtrate factor of the vitamin B<sub>2</sub> complex; these compounds do not prevent rat dermatitis. We obtained no evidence that alloxazine-adenine-dinucleotide has any nutritional activity other than that due to the riboflavin it contains. Since this coenzyme contains also adenylic acid in its molecule, these experiments also suggest that adenylic acid is not an essential nutrient for the rat; additional support for this view was provided by experiments previously published which showed that codehydrogenase II (adenine-nicotinamide-dinucleotide) had no demonstrable nutritive value for rats [Macrae & Edgar, 1937]. It is of course possible that if nicotinamide and yeast adenylic acid were required by the rat in small amounts these might be contained in our purified yeast fractions or in our basal diet in such amounts that the rats were independent of a further supply, but it is at least certain that these compounds do not replace our yeast filtrate or yeast eluate factors in the diet of the rat. Recent experiments not reported and those described above also indicate that nicotinamide and adenylic acid cannot replace the additional unidentified factor which, with riboflavin, eluate

factor and filtrate factor, satisfies the vitamin B<sub>2</sub> requirements of the rat. The question whether nicotinamide and adenylic acid are essential nutrients for the rat will be decided only when all other factors are available in a pure state.

We have made no attempt to repeat the experiments of Frost & Elvehjem [1937], who found that a combination of yeast or heart adenylic acid and nicotinamide had very marked growth-promoting properties for rats receiving a diet which differed from that used by us principally in that it contained 12 % of white corn. The possibility cannot be excluded that white corn may have some property which renders the rat capable of benefiting from the administration of adenylic acid and nicotinamide; it appears to us, however, that the introduction of such complicated materials as cereals into basal diets used in the study of the different members of the vitamin B complex renders interpretation of the results difficult.

#### C. Investigation of pimelic acid for yeast filtrate factor activity

Pimelic acid was administered in the form of its sodium salt, which we received from Dr G. H. Jeffrey. The test for filtrate factor activity was carried out as previously described [Edgar *et al.* 1938, 1]. Three rats received none of the components of the vitamin B complex except aneurin for the first week after weaning and then riboflavin and eluate factor were added for a period of 2 weeks during which their weekly weight increases averaged 16.6 and 10 g. respectively. For a further period of 2 weeks the animals then received the additional supplement of 2 mg. pimelic acid, and the average growth-rates continued to fall to 4 and 1 g. weekly. Two positive controls which received an additional supplement of yeast filtrate factor when the other animals received the pimelic acid, increased in body-weight by approximately 20 g. weekly for the period of 2 weeks during which they were observed. Pimelic acid therefore does not replace filtrate factor in the diet of the rat.

#### SUMMARY

1. Alloxazine-adenine-dinucleotide could replace riboflavin in the diet of the rat, equimolecular amounts of these compounds having the same nutritive potency. It had no demonstrable nutritive value other than its activity as a substitute for riboflavin.
2. A combination of adenylic acid and nicotinamide had no growth-promoting properties for rats receiving diets deficient in the yeast eluate factor (vitamin B<sub>6</sub>) or the yeast filtrate factor of the vitamin B<sub>2</sub> complex, and with rats deprived of eluate factor did not influence the development of dermatitis.
3. Pimelic acid did not replace yeast filtrate factor in the diet of the rat.

We wish to thank Dr H. Chick for her interest and advice, Prof. O. Warburg who kindly presented the alloxazine-adenine-dinucleotide, Prof. A. R. Todd for the aneurin, Dr G. H. Jeffrey for the sodium pimelate, Dr M. Guggenheim of Messrs Hoffmann La Roche for the riboflavin and nicotinamide, and Dr B. K. Blount of Messrs Glaxo Laboratories, Ltd., for the liver extract used in the preparation of the liver eluate fraction. The yeast used in these experiments, as in previous experiments from this laboratory, was kindly given by Messrs Watney, Coombe and Reid.

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# LXXIV. THE WATER-SOLUBLE B-VITAMINS

## XIV. NOTE ON THE YEAST ELUATE FACTOR OF THE VITAMIN B<sub>2</sub> COMPLEX

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BIOLOGICAL and chemical investigation of the yeast eluate factor [Edgar & Macrae, 1937] had indicated that this factor was probably identical with vitamin B<sub>6</sub> [György, 1935] and factor 1 [Lepkovsky *et al.* 1936], and very potent concentrates of yeast eluate factor had already been prepared when it was reported that factor 1 [Lepkovsky, 1938, 1] and vitamin B<sub>6</sub> [Keresztesy & Stevens, 1938, 1; György, 1938; Kuhn & Wendt, 1938, 1] had been obtained in a pure state.

Dr Lepkovsky very kindly presented us with a generous sample of his crystalline factor 1 and we found that this material completely replaced our yeast eluate factor in the diet of the rat. Since the biological identity of yeast eluate factor and factor 1 was thus established, we did not pursue further our independent experiments on the isolation of yeast eluate factor. However, by submitting our concentrates to the methods of fractionation found successful for factor 1 [Lepkovsky, 1938, 2] we obtained with some difficulty a small amount of a crystalline material which appeared to be identical with the hydrochloride prepared from factor 1. M.P. of our crystals, 201–203°; mixed M.P. with the hydrochloride of factor 1, 202–203°. Our crystals had the same crystalline form as that of the hydrochloride of factor 1 and also gave similar red colorations with FeCl<sub>3</sub> [cf. Kuhn & Wendt, 1938, 2; Keresztesy & Stevens, 1938, 2]. The identity of vitamin B<sub>6</sub>, factor 1 and yeast eluate factor is therefore established.

### *The activity of factor 1 in our rat growth tests for eluate factor*

Rats were prepared as previously described [Edgar *et al.* 1938, 1] and received yeast filtrate factor and riboflavin for a preliminary period of 2 weeks. Some of the animals then received an additional daily supplement of 5μg. factor 1, others 10μg. and others 20μg. In all cases increases in the growth-rate of the animals occurred (Table I). The extent by which the growth-rate of the animals was increased by administration of factor 1 was of the same order as that which has been repeatedly observed following administrations of our yeast eluate factor to rats receiving the same diet with the same supplements. Crystalline factor 1 also cured the florid type of rat dermatitis [Chick, unpublished experiments].

The growth-rate increase following administration of 20μg. daily of factor 1 (free base) was somewhat greater than that observed with 10μg. daily, which in turn was considerably greater than that observed with 5μg. daily. The optimal daily requirement of the rat for factor 1 in growth experiments appears therefore to lie between 10μg. and 20μg. daily and is probably nearer 10μg. Lepkovsky [1938, 1] observed optimal growth when he administered 10μg. of factor 1

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Table I. *Growth-promoting action of factor 1 for rats receiving a "vitamin B-free" basal diet with supplements of 10–15 µg. aneurin, 50 µg. riboflavin and yeast filtrate fraction equivalent to 1–2 g. dry yeast*

No. of rats	Av. weekly wt. increase of the group in each of 2 preliminary weeks g.	Av. weekly increase during 2 weeks g.	Factor 1 µg. daily	Av. weekly wt. increase of the group in each of 2 weeks subsequent to dosing with factor 1 g.	Av. weekly increase during 2 weeks g.
4	17, 12.5	14.7	5	20, 19.8	19.9
5	19.6, 13.6	16.6	10	26.2, 22.8	24.5
4	16.5, 14.5	15.5	20	28, 24.8	26.4
6*	18, 12	15	Yeast eluate fraction = 2 g. dry yeast	24, 25	24.5

\* Taken from a previous paper [Edgar *et al.* 1938, 2 (Table II)].

(free base) daily and Dimick & Schreffler [1939] found that 10 µg. daily produced nearly optimal growth; Kuhn & Wendt [1938, 3] stated that the rate of weight increase observed following administration of 2.5–10 µg. daily of vitamin B<sub>6</sub> hydrochloride increased with the dose of the vitamin given.

We thank Dr H. Chick for her advice and criticism and Dr S. Lepkovsky for his generous gift of a specimen of crystalline factor 1. We are grateful to Messrs Hoffmann La Roche for gifts of riboflavin.

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# LXXV. DIAPHORASE (COENZYME FACTOR)

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DIAPHORASE (coenzyme factor), one of the enzymes capable of reoxidizing reduced cozymase, has been the subject of a number of papers coming from this Institute and from Cambridge, where it was discovered independently and approximately simultaneously [Adler *et al.* 1937; Dewan & Green, 1937]. It is found widely distributed in animal tissues [Green *et al.* 1937; Dewan, 1938; Euler & Hasse, 1938], in yeast [Euler & Günther, 1938; Green & Dewan, 1938] and in several species of bacteria [Green & Dewan, 1938]. The results reported in this paper include an extension of the knowledge concerning the distribution of the enzyme in plant tissues, a further confirmation of its specificity as an oxidizing agent for coenzyme I, and a preliminary investigation of its action in respiratory systems other than those containing cytochrome carriers.

## EXPERIMENTAL

*Preparation of the enzymes.* As sources of the enzyme both plant and animal tissues were used. Four plant materials, pea and bean seedlings, the thin outer layer of common white potato and a mould culture predominantly *Rhizopus* (sp.) and one animal tissue, pig heart muscle, were treated in the following way:

The seedlings were grown in metal trays on moist blotting paper for about 12 days. The cotyledons and leaves were then removed and the stems and roots ground to a pulp in a mortar with washed sand. One volume of  $M/2$   $\text{Na}_2\text{HPO}_4$  was then added and the pulp reground until a homogeneous suspension was obtained. After an equal volume of water had been added, the coarsest particles and the sand were separated by straining through cloth and the remainder of the debris removed by centrifuging at 2500 r.p.m. for 5 min. The cloudy supernatant was treated with  $\frac{1}{2}$  vol. sat. ammonium sulphate and the precipitate so obtained separated by centrifuging. A volume of  $M/6$  secondary phosphate equal to the volume before addition of ammonium sulphate was added to the precipitate and the treatment with ammonium sulphate repeated. The precipitate finally obtained was suspended in half the original volume of  $M/6$   $\text{Na}_2\text{HPO}_4$  buffer ( $pH$  8.5). This method of preparation is a slight modification of that used by Euler & Hellström [1938] for animal tissue.

By the same procedure suspensions were prepared from potato peelings and from a mould culture which had been grown for about 2 weeks on moist bread.

The method of Euler & Hellström was used for the preparation of diaphorase from pig heart muscle. After the muscle had been reduced to a pulp and the red blood cells removed with 2 %  $\text{NaCl}$ , the decolorized mass was ground with sand and extracted with  $M/2$   $\text{Na}_2\text{HPO}_4$ . After dilution and centrifuging had been carried out for the first time, two  $\text{CO}_2$ -acetone precipitations at  $0^\circ$  were made. The precipitate obtained was suspended in  $M/6$   $\text{Na}_2\text{HPO}_4$ , treated with saturated ammonium sulphate, with  $\text{CO}_2$  and acetone and finally with sat. ammonium sulphate again. The final precipitate was suspended in  $M/6$   $\text{Na}_2\text{HPO}_4$  buffer ( $pH$  8.5).

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*Stability.* The stability of diaphorase from peas and beans differs quite markedly from that of diaphorase from pig heart. Although the latter retained its activity over a period of 4 months, the loss of activity in plant preparations was so rapid that preparations only 2 days old were unfit for experimental use. Similar observations have been made by several investigators for other materials.

*Relative concentrations of enzyme in different tissues.* It is possible only roughly to determine the relative concentrations of enzyme in plant and in animal material, since the same method of preparation cannot be used advantageously for both materials. However, when the times required for active enzymes prepared as described above to decolorize the same quantity of methylene blue in a standard Thunberg test are compared with the amount of material originally used, it is found that diaphorase in peas is only 1/6 and that in beans only 1/10 as concentrated as that in pig heart muscle. The same standard methylene blue test used to assay suspensions prepared from mould and from potatoes indicated that diaphorase was absent or nearly so from these preparations. These data are summarized in Table I.

Table I. *Concentration of diaphorase in plant and in animal tissues*

A complete system contains 0.25 ml. dihydrocozymase (1.2 mg./ml.), 0.25 ml. methylene blue (1/5000), 0.25 ml. diaphorase preparation and  $M/6$   $\text{Na}_2\text{HPO}_4$  buffer (pH 8.5); total volume 1.50 ml. All tubes were incubated at 30°

The controls indicate the spontaneous breakdown of dihydrocozymase in the presence of methylene blue and the absence of H-donating systems in the enzyme preparations.

	Material	System	Decolorization time min.	Relative concentration
1	—	Without enzyme	40	—
2	Pig heart	Complete	1.5	100
3	Pig. control	Without coenzyme	> 120	—
4	Peas	Complete	6	16
5	Peas, control	Without coenzyme	> 120	—
6	Beans	Complete	9	10
7	Beans, control	Without coenzyme	> 120	—
8	Potato	Complete	42	—
9	Potato, control	Without coenzyme	> 120	—
10	Mould	Complete	> 120	—
11	Mould, control	Without coenzyme	> 120	—

*Specificity.* The Thunberg methylene blue technique and the spectrophotometric technique were employed to investigate the specificity of plant diaphorase as an oxidant of dihydrocoenzymes I and II. The data obtained by the Thunberg method are presented in Tables IIa and IIb; those obtained by the spectrophotometric technique [Euler & Hellström, 1938] are shown in Fig. 1.

*Spectrophotometric experiments.* Exp. 1. (See below, (a) and (b).) The cell contained originally 1.0 ml. phosphate buffer (pH 7.2), 0.05 ml. diaphorase from peas, 3.7 ml. water and 0.15 ml. dihydrocodehydrase I (0.6 mg./ml.). After 10 min. 0.1 ml. methylene blue (1/5000) was added. Exp. 2. (See below, (c), (d) and (e).) The cell contained originally 1.0 ml. phosphate buffer (pH 7.2), 0.05 ml. diaphorase from peas, 0.85 ml. water and 3.00 ml. dihydrocodehydrase II (prepared enzymically). After 10 min. 0.1 ml. methylene blue (1/5000) and after 30 min. 0.1 ml. flavin enzyme were added. In both experiments the decrease in extinction at 334  $m\mu$  was measured.

(a) Dihydrocodehydrase I, diaphorase and buffer. No reaction in 10 min. No autoxidizable flavoprotein or cytochrome present.

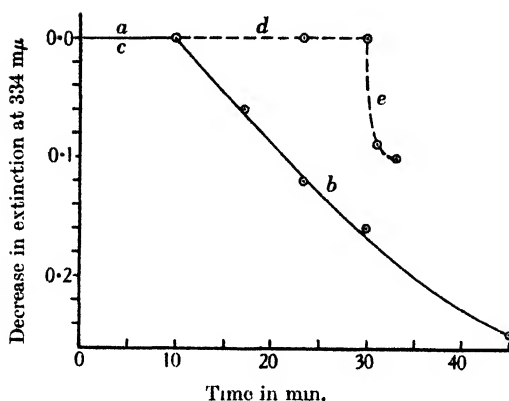


Fig. 1. Specificity of diaphorase for dihydrocodehydrase I.

Table IIa. *Specificity test. Dihydrocodehydrase I (cozymase) and diaphorase*

A complete system contains 0.25 ml. dihydrocodehydrase I (1.2 mg./ml.); 0.25 ml. diaphorase from peas; 0.25 ml. methylene blue (1/5000) and phosphate buffer (pH 8.5) to make 1.50 ml. total volume.

	System	Decolorization time, min.
1	Complete	8
2	Without coenzyme	> 80
3	Without diaphorase	37.5

Table IIb. *Specificity test. Dihydrocodehydrase II<sup>1</sup> and diaphorase*

A complete system contains (a) 0.25 ml. Robison ester; (b) 0.25 ml. Robison ester dehydrogenase; (c) 0.25 ml. codehydrase II; (d) 0.25 ml. diaphorase from peas; (e) 0.50 ml. methylene blue (1/5000); (f) phosphate buffer (pH 8.5) to make a total volume of 1.75 ml.

In tube 6 diaphorase has been replaced by 0.25 ml. flavin enzyme.

	System	Decolorization time, min.
1	Complete	28.5
2	Without (a)	> 120
3	Without (b)	> 120
4	Without (c)	> 120
5	Without (d)	28.5
6	Flavin enzyme	9.5
7	Without (a) and (b)	> 120
8	Without (a), (b) and (c)	> 120

(b) As (a) but with methylene blue added. Decrease in extinction = 0.24 unit in 35 min.

(c) Dihydrocodehydrase II, diaphorase and buffer. No reaction in 10 min. No autoxidizable flavoprotein or cytochrome present.

(d) As (c) but with methylene blue. No reaction in 20 min. No reaction between coenzyme and diaphorase.

(e) As (d) with flavin enzyme. Decrease of 0.1 unit in 3 min. Oxidizable coenzyme present.

The results of these experiments confirm the fact already reported by this Institute that diaphorase is a specific oxidant for dihydrocodehydrase I (cozymase).

<sup>1</sup> The dihydrocodehydrase II was formed *in situ* from codehydrase II and H made available from Robison ester by the action of Robison ester dehydrogenase.



*The role of diaphorase in non-cytochrome systems*

No statement has been made concerning the possible role of diaphorase in respiratory systems other than those involving cytochrome carriers. One system found in some plants both of higher and lower orders is the system in which quinones play a dominating part as H acceptors. It was felt that the best method of approach would be to study reactions involving dihydrocozymase and simple quinones, both in the presence and in the absence of diaphorase.

*Procedure.* Methods which employed the Thunberg technique and the Zeiss photometer and depended upon the disappearance of colour from solutions of quinones after the addition of dihydrocozymase were found to be inapplicable to this work. The method finally adopted depends upon the destruction of cozymase formed during the reaction between dihydrocozymase and quinone by heat in a strongly alkaline solution and subsequent measurement of residual dihydrocozymase by a fermentation technique [Myrbäck, 1933].

One set of solutions was prepared as follows: 2.0 ml. quinone (equivalent to 350  $\mu$ g. coenzyme), 0.17 ml. diaphorase and 0.13 ml. dihydrocozymase (2.6 mg./ml.). A second set of solutions contained no diaphorase but an equal volume of *M*/6  $\text{Na}_2\text{HPO}_4$  buffer (*pH* 8.5). Controls containing neither dye nor diaphorase were also prepared. One tube of the first set and a corresponding tube of the second set were allowed to react for a definite period, after which the reaction was stopped by adding 0.50 ml. *N*/10  $\text{NaOH}$ , to give a *pH* > 10. The tubes were sealed and heated in boiling water for 10 min., then the solution was brought back to *pH* 8 by adding 0.50 ml. *N*/10 acetic acid. Pairs of tubes were treated in the same manner after the reactions had continued for varying times. Two controls were carried through the same procedure, the one heated after 15 min. and the other not heated at all. By this procedure any cozymase formed during the reaction was destroyed.

A fermentation mixture corresponding to each solution to be tested for residual dihydrocozymase contained the following components: 400 mg. apozymase, 100 mg. glucose, 0.45 ml. phosphate buffer (*pH* 6.3), 0.15 ml. hexosediphosphate, 1.05 ml. water and 0.35 ml. test solution. For best results an untreated test solution should contain between 50 and 100  $\mu$ g. coenzyme per ml. Fermentations were carried out at 30° and the fermentation rate for each test solution determined from the volume of  $\text{CO}_2$  evolved during the hour of most rapid fermentation. The fermentation rate is a measure of the amount of dihydrocozymase remaining in the original solution. The results of the reaction between dihydrocozymase and three quinones, *o*- and *p*-benzoquinone and  $\beta$ -naphthoquinone, both in the presence and in the absence of diaphorase, are shown in Fig. 2.

*o*-Benzoquinone was prepared according to a modification of methods reported by Kvalnes [1934] and by Willstätter & Pfannenstiel [1904] and

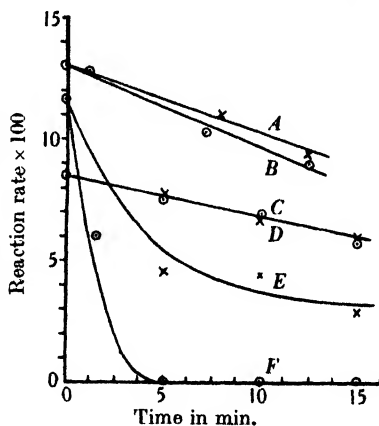


Fig. 2. Reduction of quinones by dihydrocozymase. Effect of diaphorase. Curve A,  $\times \times \times$  *o*-benzoquinone without diaphorase. Curve B,  $\odot \odot \odot$  *o*-benzoquinone with diaphorase. Curve C,  $\times \times \times$  *p*-benzoquinone without diaphorase. Curve D,  $\odot \odot \odot$  *p*-benzoquinone with diaphorase. Curve E,  $\times \times \times$   $\beta$ -naphthoquinone without diaphorase. Curve F,  $\odot \odot \odot$   $\beta$ -naphthoquinone with diaphorase.

Willstätter & Müller [1908] starting with pyrocatechol,  $\text{Ag}_2\text{O}$  and fused  $\text{Na}_2\text{SO}_4$  in absolute ether and light petroleum.  $\beta$ -Naphthoquinone was purified by recrystallization from alcohol. *p*-Benzoquinone was purified by recrystallization from alcohol and sublimation. The diaphorase used throughout the work was prepared from pig heart muscle. There was no loss of activity during the period of investigation.

The results indicate, in the cases of *o*- and *p*-benzoquinones, that diaphorase apparently plays no part in the transfer of H from dihydrocozymase to these simple quinones, but, in the case of  $\beta$ -naphthoquinone, that diaphorase accelerates H-transfer and that the reaction is extremely rapid during the first minutes. From these results no definite conclusion can be drawn concerning the part, if any, played by diaphorase in a quinone respiratory system. If, however, the result obtained with  $\beta$ -naphthoquinone can be discounted, the fact that two plant tissues (potato and mould), containing quinone respiratory systems, have little or no diaphorase parallels the observation that diaphorase did not accelerate the oxidizing action of the other quinones. A tentative conclusion, then, is that diaphorase is not necessary in respiratory systems involving quinones. This will be confirmed or denied by future investigation.

#### SUMMARY

1. Diaphorase is present in the tissues of some higher plants and absent or nearly so in those of other higher plants and fungi.
2. Plant diaphorase is extremely unstable.
3. Plant diaphorase is a specific oxidant for dihydrocodehydrase I (cozymase).
4. The role of diaphorase as H transporter in respiratory systems other than those containing cytochrome carriers is still undetermined, although tentatively it is concluded that the enzyme is not necessary in quinone systems.

The author wishes to make known his appreciation of the counsel and laboratory aid given him by Prof. H. von Euler and his assistants, Drs F. Schlenk and E. Adler. The spectrophotometric measurements were kindly made by Mr G. Günther.

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# LXXVI. EXPERIMENTS ON THE CHEMOTHERAPY OF CANCER

## III. THE INDEPENDENCE OF TISSUE RESPIRATION AND GLYCOLYSIS, AND THE GROWTH RATE OF TUMOURS

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THE main activity of tumours appears to be growth and hence it might be expected that growth rate and respiration or glycolysis would be proportional to each other under different conditions since the energy used for growth processes is probably obtained from carbohydrate metabolism. The respiration or anaerobic glycolysis of a stimulated uninjured muscle is almost proportional to the work performed [Meyerhof, 1930]. Stimulation of isolated submaxillary gland tissue with acetylcholine, which increases secretion, also increases respiration [Deutsch & Raper, 1938]. Murphy & Hawkins [1925], however, found the respiration and glycolysis of spontaneous mouse tumours to be independent of rate of growth.

The inhibition of tumour growth in rats by irradiation with X-rays is not accompanied by marked changes in glycolysis until a very considerable degeneration of tissue has occurred [Bancroft *et al.* 1935]. Some reduction of respiration may occur: in the experiments on rats given by Bancroft *et al.* the reduction is significant in about half the treated animals. In experiments of Crabtree [1932], in which rats bearing the Jensen rat sarcoma were treated with radium, an increase in the anaerobic and aerobic glycolysis of the tumour tissue was sometimes produced and the respiration was generally reduced, but growth of the tumours continued. The inhibition of growth of tumours by radiation does not appear to be necessarily dependent on inhibition of glycolysis or respiration.

In the case of muscular activity it has been shown that the decomposition of creatine phosphate is a more fundamental or essential change for activity than respiration and glycolysis. The inhibition of glycolysis with iodoacetic acid does not completely inhibit muscular activity. It now seems probable that analogous fundamental processes may govern tumour growth as creatine phosphate breakdown limits muscular action. In the present paper it is shown that the effect of some tumour-growth-inhibiting substances does not change the respiration and glycolysis of tumour tissue. In the search for compounds likely to be useful for the chemotherapy of cancer it may be considered that effective substances need not necessarily affect these processes although they are the largest changes which occur in tumour cells. It is proposed to determine which cell constituents or biochemical processes vary with growth rate or are affected by treatment with the known tumour inhibitors. It is hoped that such results may give a rational basis for further experiments in therapy.

*Growth rates and metabolism of different tumour strains.* The growth rate of different strains of transplanted tumours in mice varies from 0.01 to 1.35 mm. per day, measured as increase in average diameter [Boyland & McClean, 1935, see Table I]. Values for the respiration ( $Q_{O_2}$  from  $-7.5$  to  $-12$ ) and glycolysis

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Table I. *Growth rates and tissue metabolism of different mouse tumours*

Tumour	Mean growth rate (mm. increase in diam. per day)	Respiration	Glycolysis
		$-Q_{O_2}$ Mean (min.-max.)	$Q_L^{N_2}$ Mean (min.-max.)
S 37	1.35	11.5 (8.5-13.1)	33.5 (23.3-39.2)
Crocker 180	0.88	8 (7.2- 9.4)	23 (20.5-26.2)
M 4	0.74	8.5 (7.6-10.9)	26 (22.1-34.0)
M.C.D.B.I.	0.70	10 (9.1-11.8)	28.5 (21.7-37.5)
Mal.	0.69	7.5 (7.2- 8.5)	25 (17.7-28.5)
C 63	0.66	12 (6.2-14.4)	25 (16.5-27.5)
L.M. 154	0.032	9.5 (8.0-10.2)	22 (20.2-25.6)
Melanoma (Passey)	0.010	8.5* (5.6-11.7)	16* ( 8.9-23.7)

\* From Crabtree [1929].

( $Q_L^{N_2}$  from 16 to 34), which were determined in the usual way in the Warburg apparatus at  $37.5^\circ$ , show much smaller variations which do not run parallel with growth rate. It should be remembered that the measurements of respiration and glycolysis of isolated tissues thus determined are measurements of ability to glycolyse and respire under artificial conditions and may be different from the rates of the tissues *in situ*. The rates of glycolysis observed probably represent the maximum values, but if either the ability to respire or glycolyse were the factors limiting growth they should vary with rate of growth of the tumour.

It would be of interest to determine whether glycolysis and respiration of different tumours growing in tissue culture correspond with the growth rate of the culture. This would allow determination of growth and metabolism under the same conditions, and then all determinations would be comparable.

*Effect of treatment on tumour growth and metabolism.* Two types of tumour were used. (a) Crocker sarcoma 180 grafted into mixed stock mice in the usual way. (b) Sarcoma M.C.D.B.I. originally obtained by subcutaneous injection of 1 mg. methylcholanthrene dissolved in 0.2 ml. olive oil into a male mouse of the pure Dilute Brown strain. The tumour has always been grafted into mice of the same strain, in which it grows readily and regularly: it is now in the eighth grafted generation. A tumour of this type, which has never grown outside the same pure line of mice, resembles spontaneous or induced tumours in that genetic differences between the tumour tissue and the host's tissues are probably unimportant. In the case of the Crocker sarcoma inhibition of growth on treatment may be due either to (1) increase in resistance of the host to a foreign tissue or (2) damage to the tumour tissue *per se*. In the case of the M.C.D.B.I. tumour the factor (1) should not operate. This latter tumour has some advantages over spontaneous tumours, viz. (a) it is more easily available in large numbers, (b) tumours in different mice grow at the same uniform rate so that some mice may be used as controls, (c) the chemical constituents and biochemical reactions are probably more constant.

*Method.* As soon after grafting as possible the size of tumours was measured with calipers every second day. Sodium sulphanilyl-sulphanilate and *pp'*-diaminodiphenylsulphoxide, the inhibiting action of which has been described [Boyland, 1938] and 4:4'-diamino-2:2'-dinitrodiphenylmethane and  $\alpha$ -nitroso- $\beta$ -naphthol, which have since been found to inhibit tumour growth, were administered by daily injection into the stomachs of mice, generally commencing on the day on which the tumours were grafted. The carcinogenic substances 1:2:5:6-dibenzanthracene and 1:2:5:6-dibenzfluorene, which Haddow *et al.* [1937] and Haddow [1938] have shown to inhibit tumour growth, were injected intraperitoneally as a single dose dissolved in oil about a fortnight after

grafting the tumour. Trypan Blue (as calcium salt) and Isamine Blue (as sodium salt), both of which stain tumour tissue, were injected intraperitoneally in four or five doses of 0.25 ml. of a 1 % aqueous solution, the first doses being given about a fortnight after grafting the tumours. After a suitable interval of treatment the mice were killed and the duplicate determinations of respiration and glycolysis of the tumour tissue were made in the Warburg apparatus. Occasionally the tumour growth was not inhibited; such tumours (except those in mice treated with Isamine Blue) were not used in the measurements.

Healthy tissue free from necrotic material was used for the determinations of metabolism. No obvious difference in the extent of necrosis in normal and inhibited tumours was observed, but closer investigation of this point will be made. Tumours inhibited by 1:2:5:6-dibenzanthracene or 1:2:5:6-dibenzfluorene often appeared to have a more fibrous structure than the controls and in these cases the fibrous material was necessarily included in the samples used for experiment.

Table II. *The effect of treatment on growth rates and tissue metabolism of mouse tumours*

Mouse treated with	Dose mg.	Mean inhibition of growth %	No. of mice	Respiration ( - $Q_{O_2}$ )			Glycolysis ( $Q_{L_1}^{Na}$ )		
				mean ( $\bar{x}$ )	$\sigma$	$\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(\epsilon_1^2 + \epsilon_2^2)}}$	mean ( $\bar{x}$ )	$\sigma$	$\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(\epsilon_1^2 + \epsilon_2^2)}}$
Sarcoma M.C.D.B.I.									
Controls		—	(9)	10.5	$\pm 1.7$	—	29.6	$\pm 5.2$	—
1:2:5:6-Dibenzanthracene	15	100	(6)	10.0	$\pm 1.2$	<1	26.9	$\pm 2.2$	<1
1:2:5:6-Dibenzfluorene	5	66	(5)	7.6	$\pm 2.4$	1.4	26.6	$\pm 0.9$	1.8
Sodium sulphanilyl- sulphanilate	50	75	(6)	9.8	$\pm 0.4$	1.1	29.0	$\pm 4.2$	<1
daily									
<i>pp'</i> -Diaminodiphenyl- sulphoxide	8	50	(5)	9.5	$\pm 1.2$	1.3	23.0*	$\pm 2.5$	3.2
daily									
4:4'-Diamino-2:2'-dinitro- diphenyl-methane	8	30	(6)	10.3	$\pm 0.8$	<1	28.4	$\pm 3.3$	<1
daily									
$\alpha$ -Nitroso- $\beta$ -naphthol	8	33	(5)	9.4	$\pm 1.2$	1.4	27.3	$\pm 6.0$	<1
daily									
Trypan Blue	2.5	83	(6)	6.3*	$\pm 2.7$	3.5	22.6*	$\pm 4.7$	2.7
daily									
Isamine Blue	2.5	0	(4)	10.3	$\pm 0.4$	<1	26.2	$\pm 2.0$	1.6
daily									
Crocker sarcoma 180									
Controls		—	(9)	8.1	$\pm 0.7$	—	22.8	$\pm 2.1$	—
1:2:5:6-Dibenzanthracene	15	66	(10)	7.8	$\pm 1.5$	<1	22.5	$\pm 4.3$	<1
1:2:5:6-Dibenzfluorene	5	60	(6)	7.5	$\pm 1.4$	<1	25.4	$\pm 3.7$	1.6
Sodium sulphanilyl- sulphanilate	50	50	(5)	8.3	$\pm 0.7$	<1	24.2	$\pm 1.3$	1.4
daily									
<i>pp'</i> -Diaminodiphenyl- sulphoxide	8	67	(7)	8.3	$\pm 1.5$	<1	25.0	$\pm 2.5$	1.8
daily									
4:4'-Diamino-2:2'-dinitro- diphenylmethane	8	45	(6)	9.0	$\pm 1.1$	1.8	24.2	$\pm 2.2$	<1
daily									
$\alpha$ -Nitroso- $\beta$ -naphthol	8	50	(5)	7.6	$\pm 1.5$	<1	19.1	$\pm 3.5$	2.0
daily									
Trypan Blue	2.5	63	(7)	4.7*	$\pm 1.6$	5.3	13.2*	$\pm 6.5$	3.8
daily									
Isamine Blue	2.5	25	(5)	6.5	$\pm 1.9$	1.6	18.8	$\pm 6.5$	1.2
daily									

$\epsilon = \sqrt{\frac{\sum (\bar{x} - x)^2}{n(n-1)}}$  where  $\bar{x}$  is the mean of the series of observations,  $x$  = any individual value and  $n$  = the number of observations.  $\sigma$  = standard deviation.

\* These values are considered to be probably significantly different from the means for the corresponding control tumours—i.e. the values of  $\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}} > 2$ , where  $\bar{x}_1$  = mean value for controls,  $\bar{x}_2$  = mean value of the series under consideration.

## RESULTS

The results of this part of the experiment are given in Table II and show that with marked decrease in rates of growth, the metabolism is often not reduced. Significant reductions were found only (1) in the respiration and glycolysis of tumours in mice treated with Trypan Blue, and (2) in the glycolysis of M.C.D.B.I. from mice treated with *pp'*-diaminodiphenylsulphoxide.

## SUMMARY

1. The glycolysis and respiration of several strains of grafted tumours with growth rates varying from 0.01 to 1.35 mm. per day are not proportional to the rate of growth and vary within a narrow range.

2. The values for glycolysis and respiration of tissue from grafted tumours, the growth of which was inhibited by administration of 1:2:5:6-dibenzanthracene, 1:2:5:6-dibenzfluorene, sodium sulphanilylsulphanilate,  $\alpha$ -nitroso- $\beta$ -naphthol and 4:4'-diamino-2:2'-dinitrodiphenylmethane, were not significantly lower than values of untreated tumours.

3. The inhibition of growth produced by Trypan Blue was accompanied by decrease in respiration and glycolysis while that produced by *pp'*-diaminodiphenylsulphoxide was accompanied by reduction in glycolysis only.

4. Injection of Isamine Blue did not inhibit tumour growth nor reduce the metabolism.

We should like to thank Dr Smith of the Wellcome Chemical Works for samples of sodium sulphanilylsulphanilate and *pp'*-diaminodiphenylsulphoxide, and Prof. J. W. Cook for a specimen of 1:2:5:6-dibenzfluorene.

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# LXXVII. SOME PROPERTIES OF EGG-WHITE LYSOZYME

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*(Received 30 January 1939)*

AN enzyme capable of lysing certain bacteria, and particularly *M. lysodeikticus*, was discovered in nasal mucous by Fleming [1922] and called by him *lysozyme*. This, or a similar substance, is present in a number of animal tissues and fluids, the richest source being hen's egg-white.

An attempt to isolate lysozyme from egg-white was made by Wolff [1927] by removing inert protein with colloidal iron and precipitating the active material with acetone; the preparation obtained in this manner was said to contain no nitrogen or sulphur. Meyer *et al.* [1936] used an entirely different procedure. Acetone-dried egg white was extracted with 50 % alcohol containing 10 % acetic acid and the lysozyme precipitated from the concentrated solution with alcohol. It was purified by precipitation as the flavianate and decomposition of the latter with cold alcoholic ammonia. These investigators recognized the protein nature of the substance and considered that its lytic action involved the hydrolysis of a sugar linkage of certain mucoids present in the bacteria. The method of isolation was further improved by Roberts [1937]. Owing to the difficulty of decomposing the flavianate this part of the preparation was replaced by a fractional precipitation with acetone from a solution of the right acidity. The lysozyme was concentrated largely in the fourth and final fraction, which was said to behave in all respects like a homogeneous product, and had an activity, as determined by the method of Goldsworthy & Florey [1930], of about 2000 units per mg. This preparation was moderately soluble in water, showed most of the characteristic protein reactions, and its isoelectric point was thought to be near pH 7.0. Its dialysability through cellophane suggested that it was a protein of relatively low molecular weight.

The lysozyme used in the work described here was obtained as a white powder, dried with acetone and ether, by the method of Roberts. It had an activity of about 2000 units per mg. and contained 16.4 % N and 3.2 % S, calculated on a moisture- and ash-free basis. Ultra-centrifugal measurements by Mr J. St L. Philpot, using the absorption method, indicated that the material was homogeneous and was one of the group of proteins whose molecular weights are close to 18,000. Crystalline lysozyme was obtained from this material by Abraham & Robinson [1937] and appeared to have about the same activity as the amorphous substance, but unfortunately it has so far proved difficult to obtain crystals in sufficient quantity for chemical investigation.

In the meantime the properties of the amorphous substance, which was believed to possess a considerable degree of homogeneity, were further investigated. After an analysis had been made of certain of its constituent amino-acids it was decided to carry out an electrometric titration. Preliminary experiments in this connexion showed that the material was not completely homogeneous but that a certain fraction of it had a lower activity and much lower solubility than the rest.

## COLORIMETRIC ESTIMATION OF CERTAIN AMINO-ACIDS

*Experimental*

1.26 g. of the protein enzyme (prepared by the method of Roberts) were hydrolysed by boiling with 100 ml. of 20 % HCl for 6 hr. and most of the acid removed by evaporating the solution several times to dryness. After removing the humin by the method of Keilin & Hartree [1937] the concentrated solution of amino-acids was diluted to 100 ml. and divided into two parts (solutions A and B).

Tyrosine was estimated in solution A by the method of Folin & Ciocalteu [1927] and cystine by the method of Folin & Marenzi [1929].

Solution B was evaporated *in vacuo* almost to dryness and the residue dissolved in 50 ml. of a solution containing 10 ml. of 33 % HCl and heated on the water bath. 50 ml. of hot 15 % phosphotungstic acid solution were then added and the mixture digested on the water bath for half an hour. After standing for 2 days the precipitate of diamino-acids was filtered and washed with 100 ml. of a 7.5 % phosphotungstic acid solution containing 10 ml. of 33 % hydrochloric acid. The precipitate and filter paper were then transferred to a beaker, dissolved in just sufficient 3 % NaOH, and the solution filtered into a volumetric flask and diluted to 500 ml. (solution C).

Arginine was estimated in solution C by the method of Jean [1934] and histidine by the method of Jorpes [1932]. The values obtained were corrected for the solubilities of the phosphotungstates, using the figures given by Van Slyke [1911].

The total N in solution C was determined by the micro-Kjeldahl method and the lysine present estimated by subtraction from the total N of the arginine-, histidine- and cystine-N. To obtain a value for the latter a cystine determination was made on solution C.

*Results*

The values obtained in this manner were: arginine 11.6 %, cystine 7.0 %, lysine 5.8 %, tyrosine 4.4 %, histidine 2.6 %.

The value for cystine may be high as the starting material gave a weak positive test for sulphydryl groups [Mirsky & Anson, 1936].

Calculated for a molecular weight of 18,000 the number of basic groups per molecule, due to arginine, lysine and histidine, is about 22.

*Isolation of arginine*

1.2 g. of protein were hydrolysed with HCl and the diamino-acids separated by precipitation as their phosphotungstates in the usual manner. 150 ml. of 1 % HCl were added to the precipitate and the phosphotungstic acid extracted with 1 : 1 ether-amyl alcohol. The aqueous solution was evaporated to dryness, the residue dissolved in 15 ml. of water, heated to 90°, and 15 ml. of a solution containing 1 g. of flavianic acid added. After standing for 24 hr. in the ice chest the precipitate was centrifuged and crystallized from 110 ml. of boiling water containing a trace of flavianic acid (220 mg.). It was recrystallized from 60 ml. of hot water and dried at 100° over P<sub>2</sub>O<sub>5</sub>. (Found: N, 17.47 %. (C<sub>10</sub>H<sub>8</sub>N<sub>2</sub>SO<sub>3</sub>) (C<sub>8</sub>H<sub>14</sub>O<sub>2</sub>N<sub>4</sub>) requires N 17.2 %.)

*Van Slyke estimation of amino-N*

The estimation of free amino groups in proteins by the method of Van Slyke is complicated by the slow reaction of nitrous acid with other groups (e.g. the guanidyl group of arginine). For this reason the most satisfactory procedure



consists in plotting the  $N_2$  evolved against the time of reaction and extrapolating the final portion of the curve, due to side reactions, to zero time [Kekwick & Cannan, 1936; Rutherford *et al.* 1937]. The curve shown (Fig. 1) was obtained with a micro-volumetric apparatus. If the molecular weight of the material is

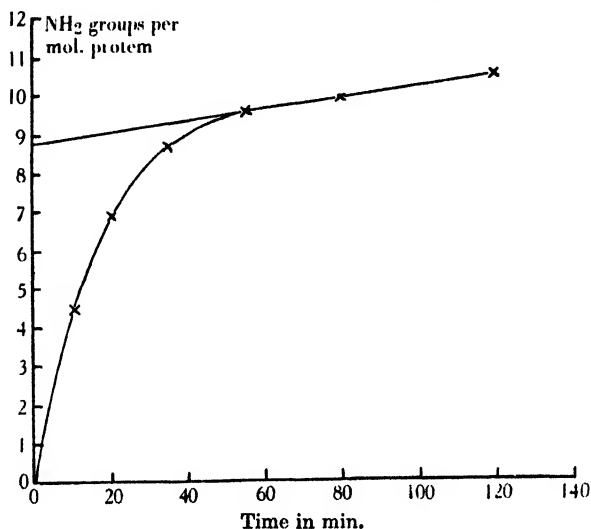


Fig. 1.

taken to be 18,000 it indicates the presence of nearly nine amino groups per molecule, and, on the assumption that only one of these is an  $\alpha$ -amino group, of eight residues of lysine. The value of 5.8%, which was previously obtained for the lysine content of the substance, corresponds to seven residues per molecule.

#### SOLUBILITY OF THE MATERIAL; ITS SEPARATION INTO TWO ACTIVE FRACTIONS

It was stated by Roberts [1937] that lysozyme was moderately soluble in water but more soluble in dilute acetic acid, being partially precipitated from the latter solution on neutralization. A closer examination of the solubility showed that this was due to the presence of a small amount of an insoluble component. The material dissolved to the extent of about 90% in ten times its weight of water, but the residue was sparingly soluble, and after washing well with water dissolved, according to a micro-Kjeldahl estimation, only to the extent of 0.024% at room temperature. Its solubility was relatively small over a wide range of  $pH$ —4.5–10.5—but it was soluble, except for a negligible residue, in 5% NaCl solution. For this reason concentrated solutions of the original substance, which had been cleared by centrifuging, became slightly opalescent on dilution. The opalescence disappeared on the addition of a drop of NaCl solution.

A further small amount of protein separated from the soluble fraction, which comprised the main bulk of the material, on dialysing its solution, in fish swim bladders, against distilled water, at 0–5°, for 3 days. After centrifuging, the activity of the protein in the resulting clear solution was not distinguishable, by the present method of estimation, from that of the original material. It was reported to be homogeneous in the ultracentrifuge and to have a sedimentation

constant of  $1.8 \times 10^{-13}$ . The protein could not be obtained from the dialysed solution by precipitation with acetone, or evaporation to dryness, without some denaturation. It was completely precipitated by ammonium sulphate between 45 and 66% saturation. Fig. 2 shows the amount of protein in solution plotted

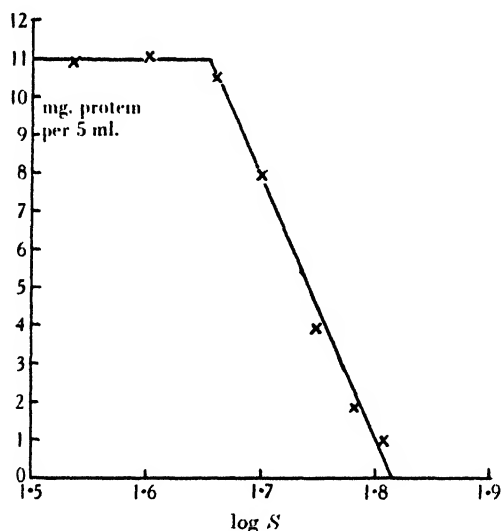


Fig. 2.

against the logarithm of the ammonium sulphate concentration ( $\log S$ ), at pH 4.6. Estimations were carried out by adding an equal volume of 5% trichloroacetic acid to the solutions, heating to  $70^\circ$  for 5 min., washing the precipitate at the centrifuge until free of ammonia, and determining its total N by the micro-Kjeldahl method.

The activity of the insoluble fraction appeared to be about one-third of that of the original substance. Ultracentrifugal measurements showed that it was less homogeneous and contained material of molecular weight lower than 18,000. Its sedimentation constant was given as  $1.63 \times 10^{-13}$ . This, together with the fact that it gave a positive nitroprusside test for sulphhydryl groups and that its nitrogen and sulphur contents (N 16.1, S 3.1%) were very similar to those of the original material, suggested that it might consist of soluble, active, lysozyme adsorbed on "denatured" lysozyme formed during the preparation. No support, however, was obtained for this view from preliminary immunological experiments which were kindly carried out by Dr B. G. Macgraith in the School of Pathology. An antiserum was obtained from rabbits injected with a solution of lysozyme from which the insoluble fraction had been removed by dialysis and centrifuging. This showed a definite precipitin reaction with the antigen solution at a dilution of the latter of  $10^4$ , but gave no reaction with the insoluble fraction at a dilution of  $10^3$ . If the latter contained an amount of adsorbed soluble protein corresponding to its activity a positive reaction would have been expected under these conditions. This preliminary result thus indicates that the lytic activity is connected with two different proteins.

## ELECTROMETRIC TITRATION

In order to obtain more information about the amphoteric nature of the preparation and the relation of the insoluble fraction to the rest of the material electrometric titrations were carried out.

*Experimental*

The apparatus consisted of a hydrogen electrode in a rocking cell, and was very similar to that used by Harington & Neuberger [1936] for the titration of insulin. It was standardized against 0.1N HCl, the pH of this solution being taken as 1.096 [Scatchard, 1925].

Solutions of the soluble protein were prepared for titration in the following way: 600 mg. of lysozyme, prepared by the method of Roberts, were dissolved in 65 ml. of water, the insoluble fraction removed by centrifuging and the clear solution dialysed in a fish swim bladder against distilled water, at 0–5°, for 72 hr. The small amount of protein which separated during the dialysis was centrifuged off, and 12 ml. of the resulting solution, containing 70–80 mg. of protein, were used for each titration. A certain amount of the material (c. 15 %) appeared to be lost during dialysis. The protein concentrations of these solutions were determined by evaporating 5 ml. portions to dryness, drying at 110°, and weighing.

The insoluble fraction was washed well with water, redried with acetone and ether, and about 75 mg. portions, in 12 ml. of water, used for each titration.

*Results*

For several reasons, but especially because of the difficulty of making adequate blank corrections, accurate titrations are usually confined to a pH range of approximately 2.5–11.5.

In the case of egg albumin [Kekwick & Cannan, 1936] and insulin [Harington & Neuberger, 1936] about 80 % of the total acid binding took place at pH 2.5, but an end-point was obtained in the first case by extending the range to below pH 2.0 and in the second case by titrating in 80 % alcohol.

In the alkaline range the situation is less satisfactory. The guanidyl group of arginine cannot be completely included in the range to which accurate titration is limited, but, nevertheless, in the titration of insulin Harington & Neuberger found that, probably because no activity correction could be made, the base binding at pH 11.5 appeared unreasonably high in comparison with the analytical data.

In the present case the blank corrections were calculated on the assumption that the concentration of hydrogen ion was equal to its activity. Under the conditions of measurement a small amount of denaturation occurred and it was not possible to keep the solutions quite clear. This was probably a surface phenomenon: Roberts [1937] remarked on the ease of surface denaturation of lysozyme. Nevertheless, it appeared unlikely that any measurable change in the acid- and base-binding capacities of the material occurred, as the titrations in both the acid and alkaline ranges were reversible. After titration, especially in the alkaline range, the material appeared to be slightly less active than it was originally, but owing to the inaccuracy of the method of estimation it was only possible to say that no great loss in activity had occurred. Lowering the temperature of titration from 25 to 20° had very little effect on the denaturation.

Fig. 3 shows titration curves of dialysed solutions of the soluble protein at 25 and 20°, and an acid titration curve in 70 % alcohol at 25°. The figures for one titration are given in Table I. The acid- and base-binding per molecule have been calculated for a molecular weight of 18,000.

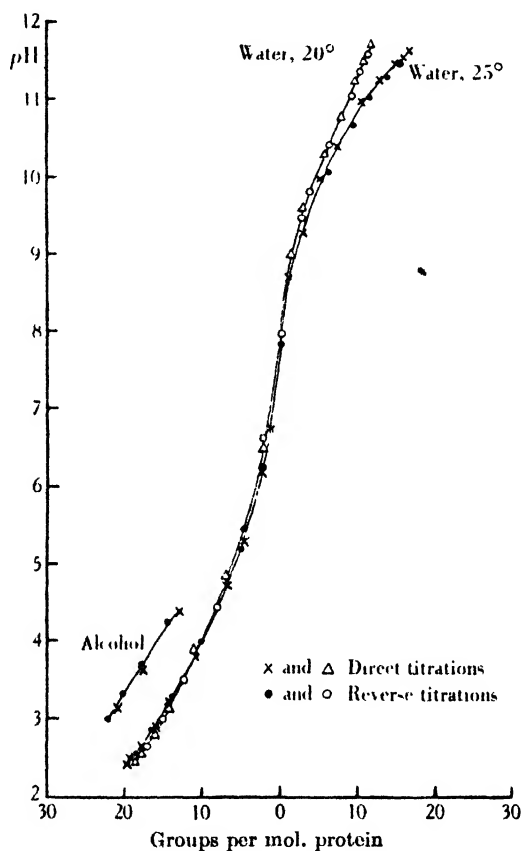


Fig. 3.

The *pH* of the dialysed solution should be close to the isoelectric point of the protein. This lay in a region where there was little buffering, and was 7.85 at 25° and 7.98 at 20°.

As would be expected the change in temperature made practically no difference to the results in the acid range. There was no indication of a maximum acid-binding at *pH* 2.5 in water, and at this point the curve shows the binding of about 19 groups per molecule of protein. It would be expected from the analytical data that there would be relatively little buffering in the range in which histidine is titrated, and in fact about 4 groups were titrated between the isoelectric point and *pH* 5.5.

Titration in 70 % alcohol was only possible in the acid range at *pH* values lower than 4.5, owing to the limited solubility of the protein in this solvent. There is difficulty concerning the definition of acidity in aqueous alcoholic solutions, and the term *pH* is used, in this case, in the sense indicated by Neuburger [1934]. The curve obtained lay about one *pH* unit above that given

Table I. *Titration of dialysed lysozyme solution*

A. With acid. 80 mg. protein (moisture and ash free); 12 ml. water. Temp. 25°.

ml. 0.1 <i>N</i> HCl	pH	ml. 0.1 <i>N</i> HCl corrected	Equiv. 10 <sup>5</sup> H <sup>+</sup> bound per g. protein	Equiv. H <sup>+</sup> bound per g. mol. protein
—	7.850	—	—	—
0.05	6.765	0.050	6.2	1.1
0.10	6.195	0.100	12.5	2.2
0.20	5.327	0.199	24.9	4.5
0.30	4.718	0.298	37.2	6.7
0.50	3.821	0.481	60.1	10.8
0.70	3.211	0.622	77.7	14.0
0.90	2.848	0.717	89.6	16.1
1.10	2.636	0.797	99.6	17.9
1.30	2.484	0.864	108.0	19.4
1.40	2.416	0.886	110.8	19.9

## Reversed titration

ml. 0.118 <i>N</i> NaOH	pH	ml. 0.1 <i>N</i> HCl corrected	Equiv. 10 <sup>5</sup> H <sup>+</sup> bound per g. protein	Equiv. H <sup>+</sup> bound per g. mol. protein
0.20	2.585	0.810	101.3	18.2
0.40	2.822	0.720	90.0	16.2
0.60	3.237	0.611	76.4	13.7
0.80	4.024	0.443	55.4	10.0
1.00	5.208	0.219	27.4	4.9
1.10	6.190	0.102	12.7	2.3

B. With alkali. 80 mg. protein; 12 ml. water. Temp. 25°.

ml. 0.118 <i>N</i> NaOH	pH	ml. 0.1 <i>N</i> NaOH corrected	Equiv. 10 <sup>5</sup> OH <sup>-</sup> bound per g. protein	Equiv. OH <sup>-</sup> bound per g. mol. protein
0.05	8.712	0.058	7.2	1.3
0.10	9.304	0.115	14.4	2.6
0.20	9.981	0.224	28.0	5.0
0.30	10.404	0.321	40.1	7.2
0.50	10.976	0.466	58.2	10.5
0.70	11.285	0.570	71.2	12.8
0.90	11.486	0.648	81.0	14.6
1.00	11.538	0.710	88.7	16.0
1.10	11.617	0.730	91.3	16.4

## Reversed titration

ml. 0.1 <i>N</i> HCl	pH	ml. 0.1 <i>N</i> NaOH corrected	Equiv. 10 <sup>5</sup> OH <sup>-</sup> bound per g. protein	Equiv. OH <sup>-</sup> bound per g. mol. protein
0.20	11.486	0.672	84.0	15.1
0.40	11.318	0.604	75.5	13.6
0.60	11.072	0.526	65.7	11.8
0.80	10.675	0.429	53.6	9.6
1.00	10.032	0.280	35.0	6.3

in aqueous solution, and at pH 3.0, where the acid-binding under these conditions would be expected to be nearly complete, the latter corresponded to 22–23 groups.

In the alkaline range no indication of maximum base-binding was obtained at pH 11.5. At this point the curve at 25° corresponded to binding by about 15 groups and that at 20° by about 11 groups per molecule of protein. This difference may be assigned to the temperature coefficients of the dissociation constants of the phenolic and guanidyl groups of tyrosine and arginine respectively.

The titration of the insoluble fraction from the original lysozyme was only possible below pH 4.5 and above pH 11. At pH 4.5 the acid-binding differed very little from that of the soluble protein, but with increasing acidity it showed greater acid-binding, the value at pH 2.5 being 24 groups per 18,000 g. At pH 11.5 the base-binding corresponded to about 21 groups. Thus at these pH values it appeared to bind about five more equivalents of acid and base than the soluble protein.

#### DISCUSSION

The colorimetric estimations showed that lysozyme prepared by the method of Roberts contained a considerable quantity of arginine and lysine and an unusually large proportion of cystine. In spite of its apparent homogeneity in the ultracentrifuge, however, the lysozyme prepared by this method is not a single protein: a certain fraction of it has a much lower solubility, a lower activity and a somewhat larger acid- and base-binding capacity, than the rest. No material is present of molecular weight higher than 18,000, but while the main, soluble, portion is homogeneous as regards molecular size, some inhomogeneity is revealed in the insoluble fraction due to particles of lower molecular weight. The latter are not present in sufficient quantity in the original material to be detected in the ultracentrifuge.

These facts would be understandable if the active proteins in both fractions were very similar in molecular size and amphoteric properties, but the insoluble fraction also contained products of partial degradation. Immunological experiments by Roberts *et al.* [1938] indicated that egg, cat and human lysozymes were not identical; but the presence of two non-identical active proteins in material obtained from one source, which is suggested by the preliminary experiments carried out in this case, is more surprising. Further experiments are required in this connexion.

#### SUMMARY

1. Lysozyme prepared by the method of Roberts appeared to be homogeneous in the ultracentrifuge and to have a molecular weight of about 18,000. A colorimetric estimation of certain amino-acids and a Van Slyke estimation of amino-nitrogen were carried out, and arginine was isolated in the form of its flavianate.

2. The solubility of the material subsequently showed that it was not completely homogeneous but that a fraction could be separated from it of lower activity and much lower solubility than the rest.

3. Electrometric titration of the soluble lysozyme showed that this was a basic protein with an acid-binding capacity not far from 23 groups per molecule. At pH 11.5 the base-binding in water, at 25°, corresponded to about 15 groups per molecule. The acid- and base-binding capacities of the insoluble fraction were somewhat larger.

The author wishes to thank Prof. W. H. Florey for the facilities he has provided; Mr Epstein for the determination of activities; and Prof. Sir R. Robinson, for his interest and encouragement.

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# LXXVIII. THE NATURE OF PHOSPHORIC ESTERS FORMED IN KIDNEY EXTRACTS

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SINCE the observation of the intense phosphorylation of glucose and glycerol in kidney extracts [Kalckar, 1937, 1] further investigations have been directed towards obtaining more information about the nature of the products of phosphorylation. The following organic substances are able to act as phosphate acceptors: adenylic acid, carbohydrates, glycerol and pyruvic acid.

## EXPERIMENTAL

*Methods.* Inorganic P was estimated colorimetrically; acid-hydrolysable P was found by subtracting the inorganic P from the P present after 180 min. hydrolysis in *N* HCl at 100°. In the tables P determined is represented by  $P_0$ ,  $P_{60}$ ,  $P_{90}$  or  $P_{180}$ , the subscript giving the number of min. of hydrolysis in boiling HCl. Triosephosphate (formed from hexosediphosphate in tissue extracts at 60°) was estimated as inorganic P after hydrolysis in *N* NaOH ( $P_{alk}$ ). Phosphopyruvic acid was estimated as inorganic P after hydrolysis in *N*/30 alkaline iodine [Lohmann & Meyerhof, 1934]; triosephosphate is not hydrolysed in *N*/30 NaOH. The adenylypyrophosphate was estimated by means of its easily hydrolysable phosphate (7 min. in *N* HCl at 100°, designated by  $P_7$ ). The fructose content was estimated by the method of Seliwanoff. The Seliwanoff reaction of fructosediphosphate is only 1/4 of that of free fructose. Dihydroxyacetonephosphate (or free dihydroxyacetone) was demonstrated by the method of Denigès [1909], i.e. instantaneous production of a purple substance in presence of conc.  $H_2SO_4$  and morphine. Pyruvic acid was estimated by the method of Clift & Cook, using the modification of Elliott *et al.* [1935]: in presence of excess  $NaHSO_3$  the pyruvic acid (and the trioses) react at room temperature to form bisulphite compounds.

The phosphorylated pyruvic acid was estimated by the  $NaHSO_3$  method after liberation of the P group by boiling in *N* HCl for 60 min. Since the keto group is combined with P, phosphopyruvic acid should not bind  $NaHSO_3$ . The addition of  $Na_2HPO_4$  in excess however will, in the presence of traces of  $I_2$ , be able to liberate a definite amount of pyruvic acid which, converting  $I_2$  into  $CHI_3$ , liberates an equivalent amount of  $NaHSO_3$ . A sample of phosphopyruvic acid (Dr Lipmann) showed the following quantitative properties:

0.1 ml. solution contained 0.044 mg. iodine-labile P and exhibited by the  $NaHSO_3$  method an  $I_2$  consumption of:

0.20 ml. *N*/200  $I_2$ , directly.

0.62 ml. *N*/200  $I_2$ , after acid hydrolysis. Calc. 0.58 ml. *N*/200  $I_2$ .

Repeated estimations on this ester all show a directly determined  $I_2$  consumption of the sample of about 0.20 ml. *N*/200  $I_2$  which is fairly independent of the absolute amounts of phosphopyruvic acid. (The preparation is free from triosephosphates). In Table XII I have therefore added 0.20 to the difference between  $P_{60}$  and  $P_0$  as a correction.



*Preparations.* I am indebted to Dr P. Ostern for a large gift of pure adenylic acid and for some of the adenylypyrophosphate used in my experiments; another specimen of adenylypyrophosphate which I prepared by the method of Lohmann was 40 % pure. The fructose-6-phosphate (Neuberg ester) was made by the method of Neuberg [1918]. Phosphopyruvic acid was made by Dr Lipmann, to whom I express my thanks. The sample prepared by the synthetic method [Kiessling, 1936] was 40 % pure.

*Experimental procedure.* An aqueous extract of kidney cortex from rabbits or cats was used. The cortex was chopped in a cooled Latapie mill and the pulp was extracted for 5 min. with an equal volume of *M*/30 phosphate buffer (*pH* 8). After the extraction the mixture was centrifuged (cold) for 1–2 min.; the supernatant liquid which was red and turbid and without cell structure was used for the experiments.<sup>1</sup> 1 or 1.5 ml. fluid was pipetted into an ice-cooled manometer vessel which contained 0.2 ml. 2 % NaF. When the cortex was extracted with water the manometer vessel contained 0.5 ml. *M*/15 phosphate buffer (*pH* 8). The side bulb contained the phosphate acceptors (adenylic acid, sugars, glycerol etc.). In most of the experiments the vessel was filled with 100 % O<sub>2</sub>, the CO<sub>2</sub> formed being absorbed by KOH. The extracts used to demonstrate the formation of unknown phosphate acceptors from malate were dialysed. The apparatus used for dialysis was a tilting board to which a bag of cellophane was tied. However, since dialysing for 3–6 hr. even at 0° very frequently destroys the ability to esterify (addition of coenzymes did not activate such extracts) the dialysis was replaced in most experiments by a washing of the chopped tissue.

### 1. Adenylic acid as phosphate acceptor

The phosphorylation of adenylic acid through anaerobic and aerobic oxidoreductions has been observed in yeast juice, muscle extracts and erythrocytes. In kidney extracts the adenylic acid is phosphorylated only under aerobic conditions. The product of phosphorylation is probably adenylypyrophosphate. The results are summarized in Table I.

Table I. *Phosphorylation of adenylic acid*

Kidney from a fasted cat, extracted with *M*/30 Na<sub>2</sub>HPO<sub>4</sub>. Per sample: 1.5 ml. extract, with the addition of 0.2 ml. 2 % NaF. Substrate: adenylic acid. Gas: 100 % O<sub>2</sub>.

	P <sub>0</sub>	P <sub>7</sub>	P <sub>7</sub> – P <sub>0</sub>
(1) Temp. 37°. Incubation: 40 min.:			
Initial sample	1.28	—	—
Incub. without substrate	0.70	0.86	0.16
Incub. with adenylic acid (1.0 mg. organic P)	0.37	1.08	0.71
(2) Temp. 25°. Incubation: 60 min.:			
Initial sample	0.88	0.93	0.05
Incub. without substrate	0.60	0.89	0.29
Incub. with adenylic acid, 18 mg. (2.4 mg. organic P)	0.08	0.88	0.80

The samples incubated with adenylic acid exhibit an enormous increase in easily hydrolysable P, indicating formation of adenylypyrophosphate. As has been demonstrated for muscle extracts and yeast juice, adenylic acid acts as a system transferring P, the adenylypyrophosphate giving its labile P to carbohydrates. Similarly the transfer of P from adenylypyrophosphate to glucose might be a step in the phosphorylation of glucose in kidney extracts. It was, however, not possible to demonstrate a transfer of P from adenylypyrophosphate

<sup>1</sup> The R.Q. of such extracts is remarkably high, about 0.9 to 1 [Kalckar, *Enzymologia*, in the press].

to glucose in kidney extracts incubated under aerobic conditions (Table II), the  $P_7$  value not decreasing in the presence of glucose.

Table II. *Addition of adenylypyrophosphate and glucose to minced kidney cortex*

Rabbit kidney, chopped. Per sample: 500 mg. tissue. Additions: 0.2 ml. 2% NaF, 0.5 ml.  $M/15$   $Na_2HPO_4$ . Substrates: glucose, 40 mg. and adenylypyrophosphate ( $P_7 - P_0 = 0.63$ ,  $P_0 = 0.30$ ). Gas: 100%  $N_2$ . Temp.  $37^\circ$ . Incubation: 60 min.

	$P_0$	$P_7$	$P_{\text{dephosphorylation}}$ (corr. for $P_0$ in "AP")
Initial sample	1.31	—	—
Incub. with adenylypyrophosphate	2.10	2.33	0.49
Incub. with adenylypyrophosphate + glucose	1.96	2.25	0.35

If  $P_7 - P_0$  at  $t=0$  was 0.63 mg.  $P$  the increases in  $P_0$  agree fairly well with the decreases in the  $P_7 - P_0$ .

It is to be noticed that the adenylypyrophosphate is dephosphorylated in the incubated samples. Since the dephosphorylation of adenylypyrophosphate is not stopped by 0.2% NaF the rate of phosphorylation of adenylic acid must be considerably higher than the rate calculated directly from Table I.

## 2. Carbohydrates as phosphate acceptors

*Glucose and fructose.* The data already reported [Kalckar, 1937, 1] on phosphorylation in kidney tissue show that glucose is phosphorylated at a considerable rate. Table III shows that fructose is also phosphorylated.

Table III. *Phosphorylation of fructose*

Kidney from cat, extracted with water. Per sample: 1.5 ml. Additions of 0.2 ml. 2% NaF and 0.5 ml.  $M/15$   $Na_2HPO_4$ . Substrate: fructose (30 mg.). Gas: 100%  $O_2$ . Temp.  $36^\circ$ . Incubation: 30 min.

	$P_0$
Initial sample	1.30
Incub. without substrate	0.94
Incub. with fructose	0.72

The investigations have been extended to include the products of the phosphorylation. The formation of hexosediphosphate has been discussed [Kalckar, 1937, 1]. The formation of triosephosphate is demonstrable in ordinary trichloroacetic acid filtrates, if the kidney extract has been incubated for some minutes at  $50-60^\circ$  immediately before the precipitation of the proteins. At this temperature a considerable part of the hexosediphosphate is converted into alkali-labile triosephosphates, mainly dihydroxyacetonephosphate. From Table IV it

Table IV. *Formation of hexosediphosphate and dihydroxyacetonephosphate*

Kidneys from rabbit, chopped, the pulp washed with 2 vol. ice water, ground with sand, extracted with  $M/15$   $Na_2HPO_4$ . Per sample: 1.5 ml. extract. Addition of NaF. Substrate: glucose (25 mg.). Gas:  $O_2$ . Incub. 30 min. Temp.  $37^\circ$ .

	$P_0$	$P_{\text{alk}}^*$	Seliwanoff (mg. fructose)	Denigès (dihydroxy- acetone)
Initial sample	1.70	—	—	0
Incub. without substrate	1.50	1.57	—	0
Incub. with glucose	0.64	0.96	1.1	+ + + + (instantaneous)

\* At the end of the experiment the incubated samples were heated to  $60^\circ$  for some minutes.

Addition of ammonium molybdate to the trichloroacetic extract of the glucose sample did not produce any laevorotatory compound (phosphoglyceric acid).

appears that the ester formed in the sample incubated with glucose exhibits the properties of dihydroxyacetonephosphate, viz. an instantaneous intense Denigès colour reaction, and liberation of P by alkali.

In attempts at isolation of the ester formed in pulp of beef kidneys, a Ba salt with the properties of hexosediphosphate was found. The Ba salt was soluble in ice water, insoluble in hot water. Liberation of P after 3 hr. boiling in *N* HCl: 60 %. Ratio H.J.-reduction/P = 1.5/1,<sup>1</sup> i.e. hexose/P = 3/1. Aldose: O. Seliwanoff: + + +. The liberation of P after 3 hr. hydrolysis is about 60 %, the liberation of P from hexosediphosphate being about 80 %. The other properties of the ester, however, agree completely with those of hexosediphosphate.

*Phosphorylation of fructosemonophosphate.* Fructosemonophosphate (Neuberg ester) added to kidney extracts acts as phosphate acceptor, yielding hexosediphosphate.

Table V. *Phosphorylation of fructose-6-phosphate*

Kidneys from cat, extracted with *M*/30 Na<sub>2</sub>HPO<sub>4</sub>. Per sample: 1.5 ml. extract. NaF added. Substrate: fructose-6-phosphate (Neuberg ester) equiv. 1.2 mg. organic P. Gas: O<sub>2</sub>. Temp. 37°. Incubation: 17 min.

	O <sub>2</sub> uptake μl.	P <sub>o</sub>
Initial sample	—	1.10
Incub. without substrate	570	0.59
Incub. with Neuberg ester	435*	0.35
Incub. with glucose (30 mg.)	540	0.22
Incub. with glucose + Neuberg ester	440*	0.21

\* The lower O<sub>2</sub> consumption in the samples incubated with Neuberg ester is due to a slight excess of oxalate after the precipitation of Ca.

As previously stated [Kalckar, 1938, 1], hexosediphosphate added to kidney extracts depresses the phosphorylation of glucose but not the phosphorylation of glycerol.

*Galactose and arabinose.* A slight phosphorylation takes place in the presence of galactose and arabinose (see Table VI). Phosphorylation of

Table VI. *Phosphorylation of galactose and arabinose*

Kidneys from cat, extracted with phosphate. Per sample: 1.5 ml. NaF added. Substrates: galactose (25 mg.) and arabinose (25 mg.). Gas: O<sub>2</sub>. Temp. 36°.

	Min.	O <sub>2</sub> uptake μl.	P <sub>o</sub>
1. Initial sample	—	—	1.22
Incub. without substrate	30	460	1.12
Incub. with galactose	30	453	0.97
2. Initial sample	—	—	1.24
Incub. without substrate	40	535	1.03
Incub. with galactose	40	610	0.88
Incub. with glucose	40	545	0.60
3. Initial sample	—	—	1.20
Incub. without substrate	60	375	1.00
Incub. with arabinose	60	380	0.88

galactose in liver has been demonstrated by Kosterlitz [1937]. Phosphorylation of ribose has not been investigated, but it appears from the recent work of Dickens [1938] that ribosephosphate is more readily metabolized than arabinosephosphate. Starch is not phosphorylated in kidney extracts (see Table VII).

<sup>1</sup> 0.121 mg. H.J.-reduction corresponds to 0.082 mg. P.

Table VII. *Addition of starch to kidney extract*

Kidneys from a rabbit, extracted with phosphate. Per sample: 1.5 ml. NaF added. Temp. 37°. Substrates: glucose (in O<sub>2</sub>). Starch (in N<sub>2</sub>).

	Min.	O <sub>2</sub> uptake μl.	P <sub>0</sub>
Initial sample	—	—	1.27
Incub. with glucose (aerobic conditions)	15	256	0.49
Incub. with starch (anaerobic conditions)	35	0	1.17

As reported [Kalekar, 1937, 1, 2], the phosphorylation of sugars is inhibited by small concentrations of phloridzin, an effect which is of importance in the discussion of the mechanism of active sugar absorption. The significance of the phosphorylation of carbohydrates will be discussed in another paper.

### 3. Glycerol as phosphate acceptor

If glycerol is added to kidney extracts under aerobic conditions an intense phosphorylation takes place (Table VIII).

Table VIII. *Phosphorylation of glycerol*

Kidneys (cat) minced, ground and extracted with phosphate. Per sample: 1.5 ml. extract. NaF added. Substrate: glycerol (20 mg.). Gas: O<sub>2</sub>.

	Min.	O <sub>2</sub> uptake μl.	P <sub>0</sub>	P <sub>180</sub>	P <sub>total</sub>
Temp. 36°:					
1. Initial sample	—	—	1.22	—	—
Incub. without substrate	40	535	1.00	1.23	1.37
Incub. with glycerol	40	580	0.66	0.89	1.32
Incub. with glucose	40	530	0.60	1.14	1.37
			P <sub>0</sub>	P <sub>90</sub>	P <sub>total</sub>
2. Initial sample	—	—	1.20	1.31	1.52
Incub. without substrate	60	275	1.00	—	—
Incub. with glycerol	60	330	0.61	0.83	1.59
Incub. under anaerobic conditions with glycerol	60	0	1.34	—	—
Temp. 22°:					
			P <sub>0</sub>	P <sub>90</sub>	
3. Initial sample	—	—	1.35	1.38	—
Incub. without substrate	60	—	0.89	—	—
Incub. with glycerol	60	—	0.52	0.78	—
Incub. with glucose	60	—	0.27	0.97	—

It will be noticed that the ester formed in the presence of glycerol is more acid-resistant than the ester formed in the presence of glucose. To determine whether the ester formed in the presence of glycerol is glycerophosphate or phosphoglyceric acid its isolation has been attempted. From 30 ml. of an extract from cat kidney incubated for 60 min. with glycerol (about 40–50 mg.) the Ba salt of an ester was prepared. Its precipitation required the addition of 3 vol. of alcohol to the neutralized trichloroacetic acid filtrate. The properties of the ester are as follows: Ba salt soluble in water, also in the presence of Ba(NO<sub>3</sub>)<sub>2</sub>, insoluble in alcohol. Quinine salt, needles, insoluble in cold, soluble in boiling water; soluble in alcohol.<sup>1</sup> A solution of 22 mg. quinine salt in 2.5 ml. alcohol in a 2 dm. tube showed  $\alpha_D = -2.78^\circ$ , i.e.  $[\alpha]_D = -158^\circ$ . The ester is very resistant to acid hydrolysis—after 90 min. hydrolysis in boiling N HCl only 1–2% of the total P is liberated. Addition of ammonium molybdate did not produce any increased laevorotation. The properties described agree completely with laevorotatory  $\alpha$ -glycerophosphate [Karrer, 1926].

<sup>1</sup> Quinine sulphate is insoluble in alcohol.

As described in another paper [Kalckar, 1939] the phosphorylation of glycerol is actually inhibited by  $\alpha$ -glycerophosphate. According to Verzar & Laszt [1934] the phosphorylation of glycerol is of importance for the absorption of glycerol and fatty acids from the intestines.

#### 4. Pyruvic acid as phosphate acceptor

Formation of phosphopyruvic acid from phosphoglyceric acid is a familiar intermediate reaction in muscle glycolysis. The formation of phosphopyruvic acid from inorganic P and pyruvic acid (formed from malic acid) is, however, a new observation.

Investigations of the action of malic acid on respiration and phosphorylation in kidney extracts led to the observation of formation of phosphopyruvic acid. If kidney extracts are dialysed for some hours and incubated with glucose a rather slight phosphorylation takes place; a sample incubated with malic acid, however, exhibits a very considerable phosphorylation (see Table IX).

Table IX. *Phosphorylations in presence of malic acid*

Rabbit kidneys, minced, extracted with 0.6% KCl solution. The extract is dialysed in cellophane. To the dialysed extract, phosphate (1.0 mg. P) and fluoride are added. Substrates: glucose and malate. Gas:  $O_2$ . Temp.  $36^\circ$ .

	Min.	$O_2$ uptake $\mu$ l.	$P_o$
Dialysis, 3 hr. at $0^\circ$ :			
Initial sample	—	—	1.10
Incub. with glucose + 0.4 mg. malate	125	338	0.89
Incub. with 10.0 mg. malate	110	885	0.38
Dialysis, 6 hr. at $-1^\circ$ :			
Initial sample	—	—	1.07
Incub. with glucose	90	115	1.11
Incub. with glucose + 10 mg. malate	90	388	0.77
Incub. with 10 mg. malate	90	387	0.81

Table IX shows that the phosphorylation in the presence of 10 mg. malic acid is very intense. Since the glycogen content of kidney extracts is very low and other carbohydrates are removed during dialysis, the intense phosphorylation in the presence of malic acid must be due to a phosphorylation of malate itself or more probably of an oxidation product of malate. The enormous increase of the respiration in the malate sample indicates an oxidation of malate to oxaloacetate. A further conversion of the oxaloacetate formed must, however, take place, since an accumulation of oxaloacetate will very soon inhibit the oxidation of malate. The  $O_2$  consumption in the malate sample is, however, remarkably constant (Fig. 1).

It is probable that the oxaloacetate is decarboxylated to pyruvate which, when phosphorylated, yields phosphopyruvate. The experiments described and others similar have given many indications that malic acid oxidized in kidney extracts yields phosphopyruvic acid.

Investigations of the ester formed in the presence of malate proved that an ester is formed which has the properties of phosphopyruvic acid [Lohmann & Meyerhof, 1934] (see Table X).

Since the extracts are frequently inactivated during 3-6 hr. dialysis even at  $0^\circ$ , most of the experiments were made with extracts from washed tissue pulp. However, "washed" extracts contain more hexoses than dialysed extracts and a formation of hexosediphosphate is unavoidable.

Table X. *Formation of an iodine-labile phosphoric ester*

Rabbit kidney cortex was minced and washed in 3 vol. ice-cooled water; the washed tissue was then ground with sand and extracted with an equal vol.  $M/30$   $\text{Na}_2\text{HPO}_4$ . 1.2 ml. extract were used. The mixture contained 0.2% fluoride. Substrates: fumarate (30 mg.), malate (20 mg.) and glucose (30 mg.). Gas:  $\text{O}_2$ . Temp.  $37^\circ$ . Incubation: 30 min.

	$P_0$	$P_{\text{alk}}$	$P_{\text{iod}}$	$P_{\text{merc}}$	$P_{30}$	$\text{O}_2$ uptake $\mu\text{l.}$
Initial sample	1.08	1.14	1.11	1.10	—	—
Incub. without substrate	0.96	1.05	1.00	1.00	1.14	230
Incub. with fumarate	0.64	0.70	0.96	0.96	1.13	432
Incub. with glucose	0.58	0.71	0.60	0.60	—	165
Initial sample (+ malate)	1.16	1.17	1.18	—	1.16	—
Incub. without substrate	1.00	1.00	1.02	—	1.11	474
Incub. with malate	0.78	0.78	0.98	1.00	1.12	720
Incub. with glucose (40 mg.)	0.75	0.83	0.77	0.75	0.96	323

$P_0$ : P determined directly.

$P_{\text{alk}}$ : P determined after 20 min. incubation in  $N/1$  NaOH.

$P_{\text{iod}}$ : P after 20 min. incubation in  $N/30$  alkaline iodine.

$P_{\text{merc}}$ : P after 10 min. incubation in neutral  $\text{HgCl}_2$ .

$P_{30}$ : P after 30 min. hydrolysis in  $N/1$  HCl at  $100^\circ$ .

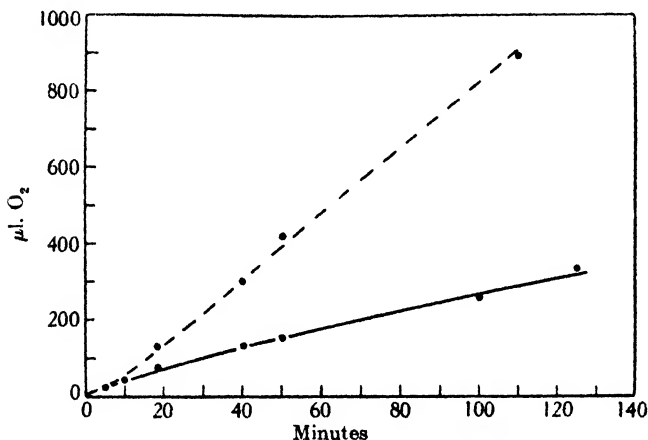


Fig. 1. Rate of  $\text{O}_2$  consumption of the incubated samples in Table X. — with 0.4 mg. malate. - - - - - with 10.0 mg. malate.

An examination of the pyruvic acid content showed that the malate samples always contain small amounts of pyruvic acid<sup>1</sup> and after 60 min. hydrolysis in boiling  $N$  HCl considerable amounts of pyruvic acid are liberated.

The difference between the contents of pyruvic acid in the samples hydrolysed with acid and the samples determined directly (and corrected, cf. Methods) gives the amount of phosphorylated pyruvic acid, a value which agrees well with that calculated from the amount of P liberated in  $N/30$  alkaline iodine (cf. Table XI).

Attempts were made to isolate the ester formed in presence of malate. In extracts of beef or cat kidneys these were never successful, probably because the preparation of extracts from such large amounts of tissue takes so long that the enzymes are inactivated. An isolation from 4 exp. with rabbit kidneys (cf. Table XI) was therefore undertaken.

The extracts from 4 exp. were combined, the total content of  $\text{I}_2$ -labile ester being about 40 mg. as Ba salt. The separation of the Ba salt of the  $\text{I}_2$ -labile ester from the Ba salts of those phosphoric esters formed in the extracts without

<sup>1</sup> No accumulation of oxaloacetic acid was detectable by the method of Ostern.

Table XI. *Liberation of pyruvic acid from the iodine-labile phosphoric ester*

I. Kidneys from a fasted rabbit are chopped, washed and extracted with phosphate. Per sample (ml. extract): initial sample 1 ml., sample incubated without substrate. 1 ml., sample incubated with malate 10 ml.\* NaF, 0.2%. Substrate: malate (150 mg.). Gas: O<sub>2</sub>. Incubation: 45 min. Temp. 38°.

	P <sub>g</sub> /ml.	P <sub>ion</sub> /ml.	P <sub>ion</sub> - P <sub>g</sub> /ml.	NaHSO <sub>3</sub> (ml. N/200 I <sub>2</sub> )	
				Direct	45 min. hydrolysis
Initial sample (15 mg. malate)	0.286	0.285	0	0.04	—
Inc. without substrate	0.230	0.230	0	0.04	—
Inc. with malate (150 mg.)	0.166	0.229	0.063	0.44	1.11

0.063 mg. I<sub>2</sub>-labile P corresponds with 0.178 mg. pyruvic acid. The NaHSO<sub>3</sub> combined with 0.178 mg. pyruvic acid consumes: 0.80 ml. N/200 I<sub>2</sub>. Found in the above exp. (1.11 - 0.44) + 0.20 (correction) = 0.87 ml. N/200 I<sub>2</sub>.

\* For isolation experiments.

II. Kidney extract (rabbit) prepared and incubated in the same manner as in exp. I. The sample with malate is incubated in 15 ml. extract. Substrates: lactate (20 mg.) and malate (20 mg.). Temp. 37°. Incubation: 45 min.

Initial sample	0.333	0.320	-0.013	0.08	0.33
Incub. without substrate	0.260	0.263	0.003	—	0.28
Incub. with lactate	0.260	0.258	-0.002	0.18	0.50
Incub. with malate	0.214	0.264	0.050	0.40	1.13

0.050 mg. P corresponds with 0.145 mg. pyruvic acid, equiv. 0.64 ml. N/200 I<sub>2</sub>. Found in exp.: (1.13 - 0.40) + 0.20 (corr.) - 0.25 (initial diff.) = 0.68 ml. N/200 I<sub>2</sub>.

III. Kidney extract (rabbit) prepared and incubated as in exps. I and II. The sample incubated with malate contains 10 ml. extract. Substrate: malate, 20 mg. Temp. 37°. Incubation: 50 min.

Initial sample	0.307	0.306	—	—	—
Incub. without substrate	0.214	0.215	—	—	—
Incub. with malate	0.142	0.214	0.072	0.32	0.92

0.072 I<sub>2</sub>-labile P corresponds to 0.205 mg. pyruvic acid. The NaHSO<sub>3</sub> compound consumes 0.90 ml. N/200 I<sub>2</sub>. Found: (0.92 - 0.32) + 0.20 = 0.80 ml. N/200 I<sub>2</sub>.

malate (probably hexosediphosphate) was, however, impossible, since the solubilities of the Ba salts were the same (insoluble in hot water, sparingly soluble in cold water, soluble in dilute acids). According to Kiessling [1936] the Ba salt of phosphopyruvic acid, like the Ba salt of hexosediphosphate, is sparingly soluble in cold water and insoluble in hot water.

The sparingly soluble Ba salt showed a satisfactory agreement between the amount of pyruvic acid determined from the NaHSO<sub>3</sub> method and that calculated from the liberation of P in N/30 alkaline I<sub>2</sub>.

From Table XII it appears that the ratio, pyruvic acid after acid hydrolysis to phosphate hydrolysed in alkaline iodine is about 3 : 1.

Table XII

For 0.5 ml. of a solution containing 15 mg. Ba salts the following values were found:

	mg. P	P <sub>ion</sub> - P <sub>alk</sub>	ml. N/200 I <sub>2</sub> (equiv. NaHSO <sub>3</sub> compound)
Directly	0.018	—	0.40
Hydrolysis in N/1 NaOH	0.020	—	—
Hydrolysis in N/30 alkaline I <sub>2</sub>	0.230	0.210	—
Hydrolysis in N/1 HCl for 60 min.	0.272	—	3.00
Control	—	—	0.02

0.21 mg. I<sub>2</sub>-labile P corresponds to 0.61 mg. pyruvic acid. 0.61 mg. pyruvic acid (NaHSO<sub>3</sub>) consumes 2.78 ml. N/200 I<sub>2</sub>. Experiment shows: 2.98 ml. N/200 I<sub>2</sub>.

Table XIII. *Identity with phosphopyruvic acid of the ester formed in presence of malate*

	Solubility of Ba salt	Alkali	Liberation of P in			Pyruv./P 3 : 1
			Iodine	HgCl <sub>2</sub>	N HCl	
Unknown P ester	Soluble in dilute acids, insoluble in hot water	No	Complete	Complete	Complete	
Phosphopyruvic	Ditto.	No	Complete	Complete	Complete	3 : 1

In Table XIII the properties of the new ester are summarized and compared with the properties of phosphopyruvic acid.

Since all properties of the new ester agree with those of phosphopyruvic acid we conclude that malic acid in kidney extracts is oxidized to oxaloacetic acid which is then decarboxylated and phosphorylated. That the phosphopyruvic acid was formed from hexosediphosphate is impossible since the intermediate product, phosphoglyceric acid, cannot be converted into phosphopyruvic acid in presence of *N*/20 fluoride. In addition no formation of phosphoglyceric acid takes place in extracts incubated with glucose.

I was never successful in bringing about a formation of phosphopyruvic acid from lactic acid,<sup>1</sup> pyruvic acid or alanine, although this might perhaps be possible in kidney and liver slices.

Without doubt the formation of phosphopyruvic acid from malic acid is related to the synthesis of sugars from the fumaric acid system. A considerable increase in the glycogen content of the livers of animals fed with fumaric or succinic acid is well established [Stohr, 1933; Blixenkrone-Møller, 1938]. In experiments with kidney slices Benoy *et al.* [1937] have demonstrated a definite formation of sugar from lactic and malic acids.

The pathway of sugar formation from lactic acid has been cleared up to a considerable extent by the studies of the Cambridge school [Green *et al.* 1937]; the understanding of the mechanism of this reaction we owe to the recent researches of the Heidelberg school [Meyerhof *et al.* 1938, 2]. Sugar formation from lactic acid is in all its properties a reversed glycolysis. Not only is the oxido-reduction the reverse of that in the glycolysis, but, as demonstrated recently by Meyerhof and co-workers, the transfer of P takes place in an exactly inverted manner. As we know, the adenylic acid in the glycolysis is rephosphorylated in two different ways: (1) through a direct P transfer from the phosphopyruvic acid (Parnas reaction); (2) through an esterification of inorganic P, a synthesis which is coupled to the reduction of cozymase by triosephosphate [Meyerhof *et al.* 1938, 1]. Very recently Lipmann [1939] has demonstrated a coupling between the reduction of aneurin pyrophosphate by pyruvic acid and the phosphorylation of adenylic acid. In the formation of sugar from lactic acid we have, in quite an analogous manner, a dephosphorylation of adenylypyrophosphate by two different reactions: (1) the direct P transfer from adenylypyrophosphate to pyruvic acid; (2) the liberation of inorganic P from adenylypyrophosphate, a reaction which is coupled to the oxidation of hydrocozymase by phosphoglyceric acid [Meyerhof *et al.* 1938, 2].

Sugar formation in animal tissues is an internal oxido-reduction which uses on a large scale the energy stored in the adenylypyrophosphate; respiration is therefore necessary for rephosphorylation of the adenylic acid. Such an

<sup>1</sup> According to Green [1936] and to Das [1937] the oxidation of lactic acid is more sensitive than oxidation of malic acid to tissue mincing.



aerobic rephosphorylation of adenylic acid has actually been demonstrated in kidney extracts.

What is the position of phosphopyruvic acid in the synthesis of sugar from lactic acid? According to Green and to Meyerhof there are three essential reactions:

(1) Lactic acid + phosphoglyceric acid + adenosinetriphosphate  $\rightleftharpoons$  pyruvic acid + phosphotriose + adenosinediphosphate + P.

(2) Pyruvic acid + adenosinediphosphate  $\rightleftharpoons$  phosphopyruvic acid + adenylic acid.

(3) Phosphopyruvic acid  $\rightleftharpoons$  phosphoglyceric acid.

Whereas reactions (1) and (3) have been demonstrated experimentally in both directions, reaction (2) has so far not been demonstrated in the direction from left to right. Probably the accumulation of phosphopyruvic acid by addition of fumaric or malic acid to kidney extracts poisoned with fluoride corresponds to reaction (2) in the direction from left to right, occurring in conjunction with an aerobic rephosphorylation of the adenylic acid. According to recent investigations [Meyerhof *et al.* 1938, 3] in which labelled P was used, it seems that the reaction (2) actually proceeds only in the direction from right to left. The enzyme system of the phosphorylation of pyruvic acid in kidney extracts therefore cannot be identical with the enzyme system of the reaction: phosphopyruvic acid + adenylic acid  $\rightleftharpoons$  pyruvic acid + adenosinediphosphate. Perhaps the oxidation of fumaric acid yields not  $\text{CH}_3\text{CO}\cdot\text{COOH}$  but another acid which is the P acceptor proper.

#### SUMMARY

1. The products formed by esterification of various P acceptors are described. Adenylic acid is phosphorylated to adenylypyrophosphate. Glucose, fructose and fructose-6-phosphate are phosphorylated to fructosediphosphate and dihydroxy-acetonephosphate. Glycerol is phosphorylated to glycerophosphate. Since the quinine salt of the phosphoric ester showed a high laevorotation,  $[\alpha]_D = -158$ , the glycerophosphate accumulated is the laevorotatory  $\alpha$ -glycerophosphate.

2. In extracts from washed tissue pulp or in dialysed extracts an intense phosphorylation very frequently takes place if fumaric or malic acid is added to the extract. These dicarboxylic acids increase the phosphorylation not only by stimulation of the respiration, but more probably by forming a phosphate acceptor. Probably a product formed by oxidation of malic or fumaric acid, must be assumed to act as phosphate acceptor. The phosphoric ester formed in the presence of malic or fumaric acid exhibits the typical properties of phosphopyruvic acid: liberation of P in  $N/30$  alkaline  $\text{I}_2$  and in neutral  $\text{HgCl}_2$ , liberation of pyruvic acid after 60 min. hydrolysis in  $N$  boiling  $\text{HCl}$ . The Ba salt of the  $\text{I}_2$ -labile phosphoric ester has been isolated in an impure state. The ratio of pyruvic acid after acid hydrolysis to P hydrolysed in alkaline  $\text{I}_2$  was about 3 : 1.

The relation of the formation of phosphopyruvic acid from malic acid to sugar formation from malic acid has been discussed.

I wish to express my thanks to Prof. Lundsgaard and Dr Lipmann for their kind interest in this work.

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# LXXIX. THE ESTIMATION OF POTASSIUM AND THE POTASSIUM CONTENT OF NORMAL VOLUNTARY MUSCLE

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POTASSIUM forms an insoluble complex with sodium, silver or lead cobaltinitrite. Kramer & Tisdall [1921] used sodium cobaltinitrite to estimate the K content of sera. Other workers [Robinson & Putnam, 1936; Truszkowski & Zwemer, 1936; Ismail & Harwood, 1937] have used silver cobaltinitrite. Ismail & Harwood, in the estimation of the K content of soils, added silver nitrate solution to the soil solution, and then Kramer & Tisdall's reagent to precipitate the complex potassium salt. The potassium silver cobaltinitrite compound they obtained was thought to consist of mixtures of  $K_2AgCo(NO_2)_6$  and  $KAg_2Co(NO_2)_6$ .

The method described below shows that it is possible to make a single precipitating reagent—a silver sodium cobaltinitrite—and that in the resulting potassium complex there is a different potassium silver relationship from any previously described.

The K content of normal muscles has been determined by this method.

*Reagent.* 22.6 g. cobalt acetate are ground up with 16.9 g. silver nitrate and placed in a 500 ml. flask; 8 ml. water and 22 ml. glacial acetic acid are added. To this are added 80 ml. water containing 44 g. sodium nitrite. After leaving overnight this solution is filtered and the filtrate aerated for 4 hr. It is stored in the ice chest and filtered prior to use.

*Method.* Serum (1 ml.) or muscle (containing from 0.1–2.0 mg. K, i.e. up to about 1–2 g. muscle) is reduced to a white ash in a silica crucible in a small electric oven at a temperature of about 350°, with sufficient conc.  $HNO_3$  to remove all halides. When the crucible is cold, the ash is dissolved in 3 ml. water; 2 ml. of this are placed in a centrifuge tube and 2 ml. of the silver sodium cobaltinitrite reagent added. After standing  $\frac{1}{2}$ –1 hr. 50 % acetone is added and the tubes are centrifuged. The supernatant fluid is decanted off and the precipitate is washed with more 50 % acetone and then finally with pure acetone. The tubes and precipitate are dried in a water bath and 3 ml. conc.  $HNO_3$  are added. After the precipitate has dissolved, some 15–20 ml. water and a little ferric sulphate are added and the tubes are placed in a boiling water bath for 2 hr. The tubes are left overnight in the ice chest and the amount of silver nitrate present is estimated by titrating with  $N/500$  potassium thiocyanate. (A tube containing a known amount of potassium nitrate can be treated in the same manner in order to check the method, although this is really unnecessary as no variation in the ratio K/Ag has ever occurred.)

Calculation:  $\frac{\text{ml. } N/500 \text{ thiocyanate used}}{5} \times \text{factor } (0.585) \times \frac{3}{2} = \text{mg. K in sample taken.}$

The method was initially tested with known amounts of potassium nitrate and it was found that the proportion of K to Ag in the compound was as 3 mol.

of the former to 2 mol. of the latter. Therefore, from this relation, a factor could be calculated which would convert ml. of thiocyanate used in the titration into mg. of K. This factor is 0.585 for *N*/100 thiocyanate. This method has been in use for the past two years and no variation in the relation has been observed.

If the sodium silver cobaltinitrite is dried, a pale brown substance is obtained which contains sodium, silver and the cobaltinitrite radicle. If this solid is re-dissolved in the appropriate quantities of glacial acetic acid and water, it yields the original reagent in liquid form.

*Solubility of potassium silver cobaltinitrite.* The solubility of the complex is 1 in 61,000 after leaving it in contact with water at room temperature for 1 week.

The almost complete insolubility of this compound is in striking contrast to that of the sodium potassium salt such as is prepared in Kramer and Tisdall's method. Some workers still use water to wash the sodium potassium cobaltinitrite precipitate, although the use of acetone is now the more common procedure. The following few examples show the differences in recovery obtained by the three methods, and it is seen that there is an increasing loss of K as one passes from the first to the last column, showing conclusively these differences in solubility.

Table I

Amount of K recovered, mg.

Amount of K taken, mg.	Author's method	Kramer & Tisdall's method	
		Precipitate acetone-washed	Precipitate water-washed
1.3	1.295	1.284	1.272
0.97	0.968	0.896	0.892
0.8	0.802	0.781	0.711
0.56	0.564	0.549	0.547

Table II

Mg. K in sample of serum taken	Mg. K added (KNO <sub>3</sub> used)	Total mg. K in serum and KNO <sub>3</sub>	Mg. K recovered	Difference mg.	% error
0.213	1.26	1.473	1.470	- 0.003	- 0.2
0.213	0.40	0.613	0.584	- 0.029	- 4.7
0.273	0.41	0.683	0.663	- 0.020	- 2.9
0.213	0.44	0.653	0.643	- 0.010	- 1.5
0.273	1.10	1.373	1.366	- 0.007	- 0.4
0.213	1.18	1.393	1.425	+ 0.032	+ 2.3
0.213	0.58	0.793	0.807	+ 0.014	+ 1.8

*Recovery experiments.* Varying amounts of potassium nitrate were added to sera of known K content and these mixtures were ashed and estimated. The recoveries are given in Table II and show that the error was usually of the order of less than 2 %.

Experiments to determine the exact composition of the K compound were made, but while the precipitate always possessed a constant composition, no simple formula could express its nature, and it is most probably a mixture of cobaltinitrites. Methods that have been used previously have yielded variable results as regards the K content of normal sera in man and this is not surprising in view of the solubility of potassium sodium cobaltinitrite. Such difficulties have been overcome by the use of the method here described, and the results obtained with normal sera have been remarkably constant. The advantages of this method are the accuracy that can be obtained in the final titration, and the high molecular weight of the precipitate, enabling time to be saved as a result of the short period needed for centrifuging.

The one disadvantage is the necessity to ash serum, but the method was originally evolved in order to estimate the K content of normal and abnormal muscle, and it is essential to ash this material irrespective of the method employed for the actual estimation.

*Potassium content of normal muscle*

Twenty-seven samples of normal voluntary muscles were obtained, mostly by biopsy, and the K contents were determined by the above method. The muscles were taken from various parts of the body. The muscle tissue was carefully dissected to remove any fibrous tissue or fat and cut up into small fragments before weighing. Drying was done in an oven maintained at a temperature of 100°, and was usually complete within 24 hr.

The average content of K was 0.28 % of wet weight of muscle, and 0.98 % of dry weight.

There was a certain degree of variation but this was not related to the situation of the muscle nor was it determined by the sex of the patient. This variation was from 0.2 to 0.35 % of wet weight, and from 0.8 to 1.2 % of dry weight.

Recently, little attention has been paid to the K content of voluntary muscle, but Leulier *et al.* [1935] found 0.4 % by wet weight in a small group of normal muscles. These workers did not dry the muscle before estimation, nor was the technique of the method employed of the same degree of accuracy as that of the foregoing method.

SUMMARY

A method for the estimation of K using a silver cobaltinitrite reagent is described, and its advantages are discussed.

The K content of normal voluntary muscles has been estimated.

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# LXXX. THE BIOLOGICAL VITAMIN D ASSAY OF LOW-POTENCY MATERIALS WITH SPECIAL REFERENCE TO THE ROLE OF THE MINERAL CONTENT OF THE DIET

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VITAMIN D is biologically determined in this laboratory by a slightly modified form of the line-test described by Morgan [1932]. Such quantities of the test substance as are known, either from the nature of the material or from the results of a preliminary trial, to contain about  $\frac{1}{4}$  or  $\frac{1}{2}$  I.U. vitamin D are given daily to each of about 10 rachitic rats. The same number of weight- and sex-paired litter-mates of these rats are each given daily doses of  $\frac{1}{4}$  or  $\frac{1}{2}$  I.U. vitamin D Standard, and the areas of the "lines" of new calcification in each group are compared. Doses of this order, i.e. between about  $\frac{1}{4}$  and  $\frac{3}{4}$ , produce, in the 10-day test-period, degrees of healing in the distal ends of rachitic radii and ulnae most suitable for quantitative assessment: quantities less than  $\frac{1}{4}$  unit produce only minute healing, while quantities greater than  $\frac{3}{4}$  unit produce massive healing which is difficult to measure and which is too close to the optimal to be critical. To prepare and administer the requisite amounts of high-potency test substances is a simple matter: the oil is diluted with refined arachis oil, so that one or two drops from a calibrated pipette have to be given to each animal daily. But if the expected potency of the test substance is less than 12.5 I.U./g. it has to be fed "neat", and if it is less than 1 I.U./g. the quantity to be fed becomes inconveniently large.

This inconvenience is particularly attendant upon the assaying of most samples of butter and many samples of margarine. Winter butter may contain less than 0.1 I.U./g. [Wilkinson, 1939], while many continental margarines are vitaminized below 1 I.U./g. (in Denmark a lower limit of 0.1 I.U./g. is stipulated by law). If such a sample is to be tested, each rat has to be given about 3 g. fat daily, a quantity which is so large a proportion of the total food consumption (normally a vitamin D test rat eats about 8–10 g. of diet daily) that the animals will not eat it all separately, and it has to be mixed into the diet. Even then it may not be all consumed.

There is an alternative procedure, namely, to extract and feed the vitamin D-containing unsaponifiable fraction of the fat. Until recently there has not been available an efficient and satisfactory method of extraction; the work of Kon & Booth [1934] indicated that some part of the vitamin D of butter fat is too unstable to pass unchanged through the necessary chemical processes. However, Bechtel & Hoppert [1936] described a method of extracting butter fat directly with alcohol and drying the alcoholic extract on a weighed quantity of diet which was then fed in measured amounts to the test animals. We have used this technique for both margarines and butters, but the results were invariably

lower than those from "direct" assays, and there was no means of ascertaining which were the more reliable. An investigation was therefore made into the reliability and limitations of the "direct" method with special reference to the effect, if any, of large quantities of dietary fat on the vitamin D therapy of rat rickets.

### *Preliminary investigations*

The simplest method of testing the reliability of an assay in which the "test substance" animals receive appreciable additions of fat is to carry out a parallel assay in which the control animals receiving vitamin D International Standard also receive equal quantities of a similar, but unvitaminized, fat. For this purpose a vegetable cooking-fat was selected and one portion of it was vitaminized to the extent of approximately 1 I.U./g. by the incorporation of a calciferol concentrate. This was the test substance. In the first assay 5 isogenic pairs of rats were used: one rat of each pair was given 0.5 g. of the vitaminized fat daily and the other 0.5 I.U. vitamin D Standard daily. In the second assay 7 isogenic pairs were used: as before the test substance was given to one half of the rats and the Standard to the other half, but the latter was also given 0.5 g. of the unvitaminized fat per rat per day. The result of the first assay was 1.25 I.U./g. and of the second, 1.1 I.U./g. These two figures are not significantly different.

To confirm directly this indication that 0.5 g. per day of extra fat does not appreciably affect the degree of healing of rickets, we then compared the areas of new calcification in two groups of isogenic pairs of rats, one of which was given vitamin D International Standard alone and the other of which was given the same dose of the vitamin with extra fat. In order to determine how much more fat could be given without disturbing the vitamin D assay, two further groups of rats were included, one of which received 2 g. fat and the other 3 g. fat per rat per day. The results are summarized in Table I.

Table I. *Apparent enhancement of vitamin D potency produced by three levels of addition of an unvitaminized cooking-fat*

No. of pairs	Daily dose of fat to one rat of each pair g.	Daily dose of vitamin D to all rats	Dose ratio:*	Apparent dose given to "fat" rats
				Actual dose given to "no-fat" rats
16	0.5	$\frac{1}{2}$ I.U.		0.935
9	2.0	$\frac{1}{2}$ I.U.		1.044
7	3.0	$\frac{1}{2}$ I.U.		1.568

\* Calculated from the standard equation: (Healing)<sup>0.7</sup> = 54.5 log dose ratio.

These results support the conclusion from the first experiment, for a dose ratio of 0.935 is not significantly different from unity; nor is the ratio 1.044, implying that even 2 g. of supplementary fat daily will not be expected to falsify an assay. The feeding of 3 g. of fat daily, however, has stimulated healing to such an extent that the apparent dose ratio has increased by over 50%. As only 7 pairs were used this figure cannot be regarded as a quantitative index of the effect of the fat: nevertheless the tendency for it to raise the ratio is unmistakable. It is pertinent to record that a few animals which received daily supplements of 2.4 g. of fat but no vitamin D showed slight "spontaneous" healing of rickets—a phenomenon never encountered when normal diets are used.

The finding that the inclusion of 30% or more of an inactive fat in a standard rachitogenic diet will enhance the therapeutic effect of vitamin D and will even induce healing (perhaps better described as apparent healing) in the absence of

the vitamin, admits of either or both of two possible explanations. The first is that the fat *per se* is responsible for the effect by increasing the absorption and utilization of Ca, thereby stimulating ossification. The second is that the effect results from the decreased consumption of the rachitogenic diet itself necessitated by the addition of the fat. The remainder of this paper is mainly devoted to a description of an investigation into the latter possibility.

### *Experimental diets and technique*

The rachitogenic diet (diet 5) in general use in this laboratory is composed of:

	%
Dried meat meal ... ..	12
Ground unmineralized flaked maize ...	84
Salt mixture ... ..	4

The relevant analytical figures are:

	Ca %	P %	Protein %
Meat meal	0.125	0.58	78
Maize	0.013	0.28	9

The composition of the salt mixture is:

	%
Calcium carbonate	75
Sodium chloride	15
Magnesium sulphate	7.5
Ferric citrate ...	2.4
Potassium iodide ...	0.1

If 38 g. fat are added to every 62 g. diet 5, there will be a corresponding fall in the percentages of maize, meat meal and salt mixture. The actual consumptions of these dietary constituents will fall even more markedly because less of the high-fat, and therefore high-calorie, diet will be eaten than of the normal diet. If the calories/g. in maize be taken as 3, in meat meal as 4 and in fat as 9, and if it be assumed that a rat normally eats sufficient food to supply 30 calories daily, the following quantities of ingredients will be ingested from the two diets:

	Total diet to supply 30 cal. g.	Meat meal g.	Maize g.	Salt mixture g.	Cooking fat g.
Diet 5	10	1.2	8.4	0.4	—
Diet D	5.7	0.42	2.97	0.14	2.17

There is thus a considerably lowered intake of protein and salt mixture, and as the latter supplies nearly all of the Ca—on the high content of which the rickets-producing effect of diet 5, as of Steenbock's diet 2965, depends—it is reasonable to expect that its deprivation is largely responsible for the enhancement of healing.

A series of experiments was then planned in which the degrees of healing produced by 0.5 I.U. daily were compared in groups of rats being fed with diets of varied compositions. For convenience of reference, the compositions of all the diets used are collected in Table II.

In all experiments does only were used. They were maintained on diet 5 from weaning (at 23 days of age), or from the day on which they reached an average weight of 45 g. until the 39th–41st day, when they were transferred to the experimental diets and maintained thereon for the 10-day test-period, at the end of which they were killed and their radii and ulnae removed.



Table II. *Compositions of diets*

Ref.	Cooking-fat %	Meat meal %	Maize %	Salt mixture %	Ca %	P* %	Ca/P* ratio	Protein %
A	33	12	46.5	8.5	2.571	0.200	12.85	13.55
B	33	12	50	5.0	1.522	0.210	7.25	13.86
C	38	12	46	4.0	1.221	0.199	6.15	13.50
D	38	7.5	52	2.5	0.766	0.189	4.05	10.53
E	33	12	53.5	1.5	0.472	0.220	2.15	14.17
5	—	12	84	4.0	1.226	0.305	4.02	16.92
F	—	8.7	88.4	2.9	0.893	0.298	3.00	14.75
G	—	18	80.5	1.5	0.483	0.331	1.46	21.49
H	—	12	87	1.0	0.327	0.314	1.04	17.19

\* The P analyses, and consequently the Ca/P ratios, are based on total P and no account has been taken of inorganic, organic, available or unavailable portions.

### Experiment 1

This was designed to investigate the effect on the degree of healing of a diet which contained 38 % additional fat but in which the meat meal and salt mixture contents remained unaltered at 12 and 4 % respectively. This was diet C. It was compared with diets 5 and D and a fourth diet, F, which contained no additional fat, but had low meat meal and salt mixture contents, viz. 8.7 and 2.9 % respectively. 27 rats were distributed between these 4 diets so that there were always litter-mate pairs of about the same weight on at least 2 diets. They were all, with the exception of 3 negative controls, given 0.5 i.u. of vitamin D standard per day. The daily food consumptions of all the animals on the experimental diets were measured. The results are assembled in Table III.

Table III. *Average daily food consumptions (F.C.) and areas of new calcification in sq. mm. (H) arranged, except those of the "no dose" rats, horizontally as weight-grouped litter-mates*

Daily dose of vit. D	Diet D			Diet C			Diet F			Diet 5		
	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H
½ i.u.	5392	5.9	434	5393	6.0	258	—	—	—	—	—	—
	—	—	—	5396	5.7	174	5397	8.1	254	5398	—	114
"	5429	6.1	286	5430	6.1	196	—	—	—	—	—	—
	—	—	—	5431	4.9	294	5432	6.5	292	5433	—	310
"	5448	6.3	378	5450	6.5	172	—	—	—	—	—	—
	—	—	—	5460	5.9	180	5461	8.1	232	5464	—	184
	—	—	—	5462	6.0	128	5463	7.3	242	5465	—	156
"	5498	5.0	162	5499	5.6	197	—	—	—	—	—	—
	5501	5.4	378									
"	—	—	—	5502	5.0	208	5504	8.4	358	5503	—	168
No dose	5391	5.4	88	5449	6.1	0	5396	7.8	146	—	—	—

In order to obtain average figures for the 4 dietary groups in the most comparable form from the above table, the following method was used: the group containing the most rats—the diet C group—was taken as a standard against which each of the other groups was compared, for which purpose litter-mate pairs only were used. As an example, the average area of new calcification (i.e. healing) of the rats in the diet F group is 275.6 sq. mm. while the average healing of their 5 litter-mates on diet C is 196.8 sq. mm., the difference being 78.8 sq. mm. Therefore, as the average healing of all the diet C rats is 200.7 sq. mm. the

relative average of the diet F rats is taken as 279.5 sq. mm. This treatment reduces the errors which arise from biological variation of healing responses in different litters and which cannot be taken into account by simple arithmetical averaging. The resultant figures are set out in Table IV.

Table IV. *Relative behaviour of the 4 groups of rachitic rats each dosed daily with  $\frac{1}{2}$  I.U. vitamin D standard. All weights expressed as g.*

	Diet D	Diet C	Diet F	Diet 5
No. of animals	5	9	5	5
Average daily food consumption	5.6	5.7	7.9	—
Average daily protein consumption	0.59	0.78	1.17	—
Average daily phosphorus consumption	0.011	0.011	0.024	—
Average daily salts consumption	0.14	0.23	0.23	—
Average daily fat consumption	2.1	2.2	0	—
Average healing in sq. mm.	337	201	279	190

N.B. In preference to Ca intakes, salt mixture intakes only have been considered throughout this work, for although it is highly probable that Ca carbonate is the important variant, nevertheless the presence of the other salts in constant proportions theoretically prohibits the making of any deductions about Ca alone.

The only observation that can be made from this table is that the rats consuming the least quantity of salt mixture—those on diet D—displayed most healing. There is no indication of the existence of a simple relation between cooking-fat consumption and degree of healing. Both undosed animals on the low-salt diets showed some healing.

### Experiment 2

This was designed to confirm the findings of Exp. 1 and concomitantly to ascertain to what extent the degree of healing produced on a high-fat diet could be controlled by variation of the intake of salt mixture. The levels of inclusion of salt mixture chosen were 1.5, 5 and 8.5% in a diet containing meat meal,

Table V. *Average daily food consumptions (F.C.) and areas of new calcification in sq. mm. (H) arranged, except those of the "no dose" rats, horizontally as weight-grouped litter-mates*

Daily dose of vit. D	Diet E			Diet B			Diet A			Diet 5		
	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H
$\frac{1}{2}$ I.U.	6629	8.5	930	6630	7.0	146	—	—	—	—	—	—
"	—	—	—	6633	6.8	208	—	—	—	6631	12.1	280
"	6635	7.0	456	6636	7.5	192	6637	7.7	100	—	—	—
"	6673	7.0	342	6671	8.1	296	6672	9.1	240	—	—	—
"	—	—	—	6816	6.7	122	—	—	—	6815	12.7	322
"	6819	6.5	464	6820	7.6	90	—	—	—	—	—	—
"	—	—	—	6751	5.4	356	—	—	—	6750	13.3	202
"	6748	7.0	574	6748	9.1	185	6752	6.6	406	—	—	—
"	—	—	—	6829	6.6	218	—	—	—	6830	10.9	150
"	6834	6.0	336	6832	9.0	130	—	—	—	—	—	—
"	—	—	—	6836	8.2	116	6837	8.8	96	—	—	—
"	8451	7.2	222	8449	6.2	113	8452	7.0	81	8450	8.4	122
"	8460	8.2	391	8404	7.0	167	8462	7.3	39	—	—	—
"	8466	8.4	243	8465	6.8	138	8469	7.6	86	8468	7.6	180
No dose	6632	6.1	522	6638	6.9	0	—	—	—	6634	7.9	0
"	6818	5.7	177	—	—	—	—	—	—	6817	10.6	0
"	6833	4.4	242	6835	6.1	0	—	—	—	6828	8.9	0
"	8467	5.9	190	—	—	—	—	—	—	6831	9.0	0
"	8545	6.9	110	—	—	—	—	—	—	—	—	—
"	8581	5.8	158	—	—	—	—	—	—	—	—	—

maize and 33 % cooking-fat. These were designated diets E, B and A respectively. Control animals were maintained on diet 5 and this time all food consumptions, including those of the diet 5 animals, were measured. On each diet a few animals were maintained without vitamin D doses. In all other particulars the experimental details were the same as before. The data from this experiment, in which 48 rats were used, are collected in Table V.

The comparative figures for the several dietary groups, obtained as explained previously, have been arranged in Table VI.

Table VI. *Relative behaviour of the 4 groups of rachitic rats, each dosed daily with 0.5 I.U. vitamin D. All weights expressed as g.*

	Diet E	Diet B	Diet A	Diet 5
No. of animals	9	14	7	6
Average daily food consumption	7.0	7.3	7.1	11.7
Average daily protein consumption	0.99	1.01	0.97	1.98
Average daily phosphorus consumption	0.015	0.015	0.014	0.036
Average daily salts consumption	0.11	0.37	0.61	0.47
Average daily fat consumption	2.3	2.4	2.4	0
Average healing in sq. mm.	455	177	154	194

Again it appears that salt mixture intake and degree of healing are the important variants. A salient feature of the results is the massive healing displayed by the rats which were consuming the lowest level of salt mixture. The average daily salt mixture consumption of these rats, 0.11 g., is about one-fifth of the normal and the areas of new calcification were so large that accurate assessment was difficult. Moreover, the 3 undosed rats receiving diet E showed considerable "spontaneous" healing. The figures in Table VI do not suggest that the excessive dietary fat has any appreciable direct effect on the healing. It will have been observed, however, that the tabulated results of the last two experiments indicate the existence of some correlation between healing and one other factor, viz. the Ca/P ratios of the diets. As the P contents do not vary within wide limits, it is to be expected that the Ca/P ratios will closely follow the Ca contents: this aspect will be considered in detail later.

### Experiment 3

The next experiment was on a smaller scale. Its object was to discover whether spontaneous healing of rickets could be effected by feeding a diet containing very small quantities of salt mixture but no additional fat. For this purpose diet H was made up. As the animals would eat more of this diet than of the high-fat diet E, the salt mixture was included at only 1 %. Some of the rats received vitamin D and some received none. Table VII contains the relevant data. Food consumptions were not recorded.

Table VII. *Production of "spontaneous" healing of rickets by the feeding of a low-salt diet for 10 days*

	Diet 5 (undosed)	Diet 5 ( $\frac{1}{2}$ I.U./day)	Diet H (undosed)	Diet E (undosed)
No. of animals	2	2	3	3
Average healing in sq. mm.	0	36	82	117

Extensive healing has taken place on the low-salt diet in the absence of either fat or vitamin D. It is impossible, with so few rats, to state whether or not the difference between the healings on diets H and E is significant. A more elaborate comparison of the two diets was then conducted.

*Experiment 4*

To compare with diets H, E and 5, a new diet, diet G, was introduced, which contained no added fat, only 1.5 % salt mixture, but 18 % meat meal; its purpose was to determine the effect of increasing the protein intake of the animals. The conduct of the experiment conformed closely to that of the three main experiments reported above, and the results are presented in a similar form in Table VIII.

Table VIII. *Average food consumptions (F.C.) and areas of new calcification in sq. mm. (H) arranged, except those of the "no dose" rats, horizontally as weight-grouped litter-mates*

Daily dose of vit. D	Diet E			Diet H			Diet G			Diet 5		
	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H
½ I.U.	—	—	—	8538	9.4	480	—	—	—	8537	10.6	125
"	—	—	—	8539	9.7	480	—	—	—	8541	10.9	150
"	—	—	—	8540	10.9	463	—	—	—	8542	10.3	166
"	—	—	—	8602	8.2	530	8598	9.0	320	8601	8.1	173
"	—	—	—	—	—	—	8600	9.6	451	—	—	—
"	—	—	—	8614	11.6	444	—	—	—	—	—	—
"	—	—	—	8617	11.6	537	8616	11.4	539	8615	10.8	257
"	—	—	—	8619	8.4	543	8618	10.2	442	—	—	—
"	—	—	—	8644	10.2	492	—	—	—	—	—	—
"	8648	7.5	411	8645	10.6	586	—	—	—	—	—	—
"	8690	8.2	416	8689	10.6	332	—	—	—	—	—	—
"	—	—	—	8692	8.1	413	—	—	—	8691	9.9	246
"	8702	7.7	423	—	—	—	—	—	—	—	—	—
"	8703	7.5	407	8701	10.4	410	—	—	—	—	—	—
"	8731	8.9	372	8733	11.3	466	—	—	—	—	—	—
"	8734	8.5	353	8736	10.3	462	—	—	—	—	—	—
"	8768	7.3	418	8769	9.4	364	—	—	—	—	—	—
No dose	8730	6.3	163	8599	7.4	172	8597	6.7	142	8643	10.7	0
"	8732	6.3	211	8770	8.2	326	—	—	—	—	—	—
"	8735	6.9	130	—	—	—	—	—	—	—	—	—

By the usual method the group averages from these data have been collected in Table IX.

Table IX. *Relative behaviour of the 4 groups of rachitic rats, each dosed daily with 0.5 I.U. vitamin D. All weights expressed as g.*

	Diet E	Diet H	Diet G	Diet 5
No. of animals	7	15	4	6
Average daily food consumption	7.6	10.1	10.8	10.4
Average daily protein consumption	1.07	1.73	2.33	1.76
Average daily phosphorus consumption	0.017	0.032	0.036	0.032
Average daily salts consumption	0.114	0.10	0.16	0.42
Average daily fat consumption	2.5	0	0	0
Average healing in sq. mm.	431	467	406	181

It will be noted that neither the protein nor the fat consumption has any manifest bearing on the degrees of healing.

## DISCUSSION

All the experiments that have been described have yielded consistent results and the three major experiments (nos. 1, 2 and 4) in particular appear to be mutually supporting. In order to obtain a composite picture of the results they

have been re-tabulated below in dietary groups placed in order of the average degree of healing of rickets.

Table X. *Collected data concerning healing of rickets and diet. (All rats dosed  $\frac{1}{2}$  I.U. vitamin D for 10 days; all weights expressed as g.)*

Exp. no.	No. of rats	Diet	Av. healing in sq. mm.	Av. daily consumption of				Ca/P ratio of diet
				Salt mixture	Phosphorus	Protein	Cooking-fat	
4	15	H	467	0.10	0.032	1.7	0	1.04
2	11	E	455	0.10	0.015	0.99	2.3	2.15
4	7	E	431	0.11	0.017	1.1	2.5	2.15
4	4	G	406	0.16	0.036	2.3	0	1.46
1	5	D	337	0.14	0.011	0.59	2.1	4.05
1	5	F	279	0.23	0.024	1.2	0	3.00
1	9	C	201	0.23	0.011	0.78	2.2	6.15
2	6	5	194	0.47	0.036	2.0	0	4.02
4	6	5	181	0.42	0.032	1.8	0	4.02
2	14	B	177	0.36	0.015	1.0	2.4	7.25
2	9	A	154	0.60	0.014	0.97	2.4	12.85

Of the five dietary variants listed, three, namely, phosphorus, protein and fat, are obviously unrelated to healing, while the other two, salt mixture intake and Ca/P ratio tend to increase with decrease of healing. The variation of the Ca/P ratio is of approximately the same order as that of the salt mixture intake; and this is to be expected because (a) the phosphorus, being wholly supplied by the maize and meat meal, does not vary widely, and (b) salt mixture intakes are not markedly disproportional to salt mixture contents of the diets. Although from the practical standpoint the ratio is unimportant, and will not therefore be taken into consideration, theoretically it merits close attention [cf. Bethke *et al.* 1932; Shohl, 1936; 1937]. To investigate the true role of the Ca/P ratio, diets with constant Ca contents and variable P contents must be included; moreover, allowance must be made for the presence of such unavailable forms of P as inositolhexaphosphate [Bruce & Callow, 1934, 2]. In the preceding Tables total P only has always been given and no account has been taken of the availability of the element. Consequently the Ca/P ratios given are probably lower than the more important Ca/available-P ratios.

For the purpose of graphical presentation of the relation between the two variants the collected results were divided into 4 groups, which were:

- (i) rats receiving  $\frac{1}{2}$  I.U. daily and supplementary fat,
- (ii) rats receiving  $\frac{1}{2}$  I.U. daily and no supplementary fat,
- (iii) and (iv) the undosed rats with and without supplementary fat respectively.

The healings of each group were then arranged in order of intensity and marked off into subgroups each representing about 8 rats. Average healing and salt intake were calculated for each subgroup and the resulting figures plotted. Necessarily it was impossible to take into consideration weight- or isogenic-grouping, but the large number of animals used (there were 131 altogether, including 20 odd "unpaired" rats on the several diets which could not be incorporated in the separate experimental results) would cancel any errors due to variability of litter responses. The resultant averages were used to construct the curves in Fig. 1, which clearly illustrate both the lack of effect of the added fat and the relation of rickets healing to salt mixture intake with and without the presence of vitamin D.

The requisite adjustment of salt mixture intakes in an assay involving the feeding of large quantities of fat can be carried out in any of three ways:

- (a) indirectly by equalization of fat percentages in all diets,
- (b) directly by the equalization of the salt mixture in the diets as parts per anticipated daily consumption,
- (c) directly by the equalization of the salt mixture in the diets as parts per calorie.

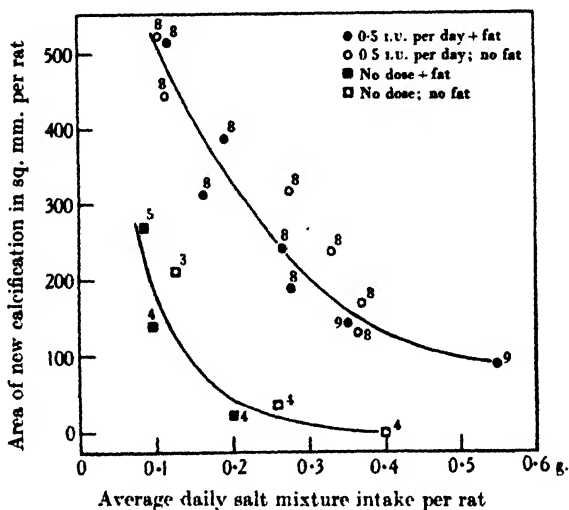


Fig. 1. The small figures indicate the number of rats represented by each point.

Because the primary object of this investigation was to determine the best method of assaying low-potency butters and margarines, all experiments have been based on the standard assay technique with its 16-18 day rachitogenic period on "diet 5". The ancillary derivation of the relation between degree of healing and salt mixture intake cannot therefore be directly compared with similar derivations made by other workers who have usually fed their experimental diets from weaning. It is interesting to note, however, that Querido [1935] fed to rats a diet containing 0.35 % Ca and 0.35 % P (i.e. similar to our diet H, which gave very heavy healing) and found that it was non-rachitogenic even in the absence of vitamin D. Bruce & Callow [1934, 1] showed that the addition of available P to a high Ca/P diet markedly increased the therapeutic efficacy of vitamin D dosing.

One aspect of rickets has been left untouched, viz. the composition of the bone ash. The effect of various salt mixtures on this will be examined in a subsequent publication.

#### SUMMARY

Experiments are described in which rachitic rats have been dosed with equal quantities of vitamin D in conjunction with various levels of the standard rachitogenic salt mixture, and, in some cases, with large quantities of fat. It has been found:

- (1) that, under the conditions of experimentation in this laboratory, the presence of excess fat in the diet influences the degree of new calcification produced by vitamin D only indirectly by lowering the consumption of salts. In assay practice this means that low-potency butter and margarines can be

accurately tested only by equalization of the salt mixture intakes of test substance and "standard" rats;

(2) that the healing of rickets produced by vitamin D, as measured by the line test, is inversely proportional to the intake of salt mixture of which Ca is probably the influential constituent, and that a very low salt mixture intake will produce healing in the absence of vitamin D.

The authors wish to thank the Directors of Lever Brothers & Unilever Limited for permission to publish these results.

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# LXXXI. THE PHYSIOLOGICAL PROPERTIES OF ASCORBIC ACID

## III. EFFECTS UPON WATER BALANCE AND UPON BODY COMPOSITION OF GUINEA-PIGS

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*(Received 18 March 1939)*

IN the first paper of this series a weight difference caused by ascorbic acid in paired-fed guinea-pigs was reported [McHenry *et al.* 1938]. Several explanations regarding differences in metabolism, water balance and food absorption were suggested, and, in a later paper, observations on a difference in O<sub>2</sub> consumption were described [Fidlar *et al.* 1939]. The present communication deals with differences in water balance and in body composition under conditions similar to those previously used.

Comparatively few references dealing with the effects of ascorbic acid upon water balance and upon the amounts of protein, fat and ash in guinea-pigs have been found in the literature. Doi [1938] reported an increase in the water content of liver, kidney, lung and muscle in scurvy. There are various reports on changes in nitrogen metabolism or in plasma protein [Doi, 1938; Nagayama & Sato, 1928; Shipp & Zilva, 1928] but none have been found regarding changes in the total nitrogen content of the animal. Doi [1938] found that the ash content of scorbutic guinea-pigs was normal. Other reports deal only with bone ash. The content of fatty acids was reported by Nagayama & Tagaya [1929] to be lower in scorbutic animals than in normal ones.

Many of these investigators have pointed out that inanition may have been a factor in causing the observed results since there is a progressive loss of appetite as animals become deficient in ascorbic acid. We have endeavoured to eliminate the factor of inanition by using paired feeding, although *ad lib.* feeding has also been employed to study the effect of inanition.

### METHODS

The technique of paired feeding employed by us, the care of animals and the composition of the basal diet have been described previously [McHenry *et al.* 1938]. As before, 3 groups of guinea-pigs were used, a "normal" group given basal diet *ad lib.* plus 5 mg. ascorbic acid daily, a "basal" group given basal diet *ad lib.* and a "paired" group receiving that amount of basal diet consumed by the basal group on the previous day, plus 5 mg. ascorbic acid daily. The animals were housed in individual metabolism cages and received water *ad lib.*; daily records were kept of the intake of water and excretion of urine.

At the end of 21 days the animals were killed and the bodies prepared for analysis by freezing on CO<sub>2</sub> ice. The frozen bodies from each group were finely minced and then thoroughly mixed. From the mixed material aliquots were withdrawn for moisture determinations, conducted by drying to a constant weight at 50°.



After drying, the aliquots were reground and mixed, after which fresh aliquots were used for determinations of fat, nitrogen and ash. Total nitrogen was estimated by the Kjeldahl procedure, fat by ether extraction in a Soxhlet apparatus and ash by ignition to constant weight in an electric furnace.

### RESULTS

Observations were made on three series totalling 15 pairs of animals and on two series of 10 normal guinea-pigs. Results from one series only are given since those from all series were comparable.

Table I gives the observations on water balance. All figures are averages for groups of five animals.

Table I

Group	Total water intake ml.	Total urinary excretion ml.	Difference ml.	Body-water g.	Body-wt. g.
Normal	946	189	757	213	293
Basal	869	286	583	163	222
Paired	1260	542	718	186	248

Table II

	Normal		Basal		Paired	
	%	Wt. g.	%	Wt. g.	%	Wt. g.
Water	72.8	213.0	73.6	163.0	74.9	186.0
Fat	4.5	13.2	3.6	8.1	1.9	4.9
Protein	16.6	48.8	18.0	38.9	16.4	40.7
Ash	3.4	10.0	3.7	8.3	3.0	9.8
Total	97.4	285.0	98.9	218.3	97.3	241.4
Body-water		293		222		248

Table II shows the results of the nitrogen, fat and ash determinations. The body-water content is repeated for comparison. Nitrogen content is given as protein, calculated with the conventional factor of 6.25. Values are given in weight as well as percentages since there are definite differences in the body weights of the separate groups.

### DISCUSSION

Paired feeding has been used by us, as it has been employed by many others, to restrict the effect of differences in food consumption. The basal and paired groups received isocaloric amounts of basal diet and, so far as other foodstuffs were concerned, differed from each other only in supplies of ascorbic acid and in the amount of water consumed. The normal group had available considerably more food. Records of food consumption show that the total food consumption of the basal and paired groups was 368 g. per animal, while that of the normal group was 443 g. The general interpretation of the experiment carried out in this way is that the basal and paired groups differed only in respect of the supply of ascorbic acid and that any observed results were due to this single variable.

Considerable differences in water intake and urinary excretion were observed among the three groups. Although both the intake of water and output of urine in the paired group were much greater than in either of the other groups the water retention of the paired animals was of the same order as that of the normal group. The increased water consumption of the paired-fed guinea-pigs may have been due to their unsatisfied hunger because of the restricted food intake.

Ascorbic acid is apparently concerned, directly or indirectly, with water retention. These results show that the weight differences observed in paired feeding experiments previously reported from this laboratory [McHenry *et al.* 1938] can be largely explained by variations in water retention. 71% of the difference in body-weight between the normal and basal groups is due to water, while 88 % of the difference between the weights of the basal and paired groups is due to water.

The decreased amounts of fat and protein found in the paired animals in contrast to those fed *ad lib.* would be expected because of the restricted supply of food but there is no difference in the total amount of ash. Animals in the paired group have not a normal body composition. While the amounts of protein and ash are less in the basal group than in the paired animals the differences are slight. There is one marked distinction between the basal and paired animals, namely, the amount of fat in the bodies. Guinea-pigs on a scorbutic diet have a greater  $O_2$  consumption than paired animals given ascorbic acid, yet they retain almost double the amount of fat. We have no explanation at present for this difference in total body fat.

#### SUMMARY

Water intake, urinary excretion, and the amounts of water, fat, protein and ash have been studied in guinea-pigs receiving a basal scorbutic diet, in animals given isocaloric supplies of the diet plus ascorbic acid and in animals receiving the vitamin but allowed to eat *ad lib.* Under these conditions paired guinea-pigs drink more water and excrete more urine than do animals in the other groups. A lack of ascorbic acid causes a diminished retention of water which largely accounts for differences observed in body-weight. The deficient animals retain considerably more body fat than do the paired guinea-pigs despite the increased  $O_2$  consumption which has been reported previously.

This investigation has been greatly facilitated by a grant from the Banting Research Foundation for which the authors are sincerely grateful.

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# LXXXII. THE BIOLOGICAL DETERMINATION OF CRYSTALLINE VITAMIN B<sub>1</sub>

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KINNERSLEY & PETERS [1936] stated that, although they had always found a rough relation between effect and dose of vitamin B<sub>1</sub> when this was given in the form of a food or concentrate, this relation did not hold at all when crystalline vitamin B<sub>1</sub> was given. They measured effect by the duration of the cure of "retracted neck" in pigeons. Doses of concentrates were given by mouth but doses of crystalline vitamin B<sub>1</sub> were given by injection. When they gave doses of crystalline vitamin B<sub>1</sub> by mouth, the effect, as measured by the duration of cure, still bore no relation to the size of dose given, but when measured by the percentage of birds cured (7  $\mu$ g. cured 75 % and 14  $\mu$ g. cured 90 %) then a relation between effect and size of dose became apparent. They therefore considered the oral route the more satisfactory way of giving doses, but concluded finally that the "day-dose" method of evaluating results from pigeons is not satisfactory for assay of injected crystalline vitamin B<sub>1</sub> though useful in assaying impure preparations of the vitamin.

We had ourselves by that time obtained one result showing a relation between effect and dose of crystalline vitamin B<sub>1</sub>, the effect being measured by the percentage of birds cured and doses being given orally. There was also a difference in the effects as measured by the average duration of the cure but it was not a large one (Table Ia). We decided, therefore, to carry out further tests, as it seemed probable that crystalline vitamin B<sub>1</sub> would shortly take the place of the adsorbate of that factor which had been used as the International Standard for several years. We wished also to obtain further information on the accuracy of the pigeon test, for it has the distinct advantage of being specific for vitamin B<sub>1</sub> while the "increase in weight" method is not.

## EXPERIMENTAL

We always use pigeons suffering from their first attack of "retracted neck". Pigeons which have been cured in one experiment are never used a second time. As the birds become ready for test they are distributed in rotation to the various groups arranged for the comparison, so that as nearly as possible equal numbers of birds are assigned to each group and given their doses on the same day.

Our first experiment, mentioned above, with graded doses of the crystalline vitamin B<sub>1</sub>, was a comparison between the crystalline preparation destined for the new International Standard and the current International Standard of vitamin B<sub>1</sub> adsorbate. In all experiments doses were given orally. In this one, doses of 5 and 10  $\mu$ g. crystalline vitamin B<sub>1</sub> were tested in series with a dose of 3 units (0.03 g.) of the International Standard. The dose of 5  $\mu$ g. produced 33.3 % of cures in 15 birds and the dose of 10  $\mu$ g. produced 66.7 % of cures in 15 birds. This was very clear evidence of a graded response to graded doses of the vitamin (Table Ia).

Table I. *Response of pigeons to graded doses of crystalline vitamin B<sub>1</sub> given orally. Criteria: percentage of birds cured and duration of cure*

Exp.	Dose μg.	No. of birds dosed	% birds cured	Av. duration of cure of birds, days
a	5.0	15	33.3	3.6
	10.0	15	66.7	4.3
b	4.5	22	54.5	6.6
	9.0	22	81.8	5.5
	13.5	22	90.9	6.2
c	3.0	22	59.1	6.2
	6.0	22	50.0	8.7
	9.0	22	77.3	7.4
d	1.0	25	8.0	5.5
	3.0	25	28.0	5.6
	9.0	25	56.0	5.4

Our second experiment was performed merely to investigate the nature of the relation between the dose of crystalline vitamin B<sub>1</sub> given by mouth and the percentage of birds cured. Doses of 4.5, 9.0 and 13.5 μg. of crystalline B<sub>1</sub> were given to different groups, each group consisting of 22 birds. The percentages of birds cured were 54.5, 81.8 and 90.9 respectively, again a graded response to graded doses given (Table Ib).

In our third experiment doses of 3, 6 and 9 μg. respectively were given to different groups with 22 birds in each group. The percentages of birds cured were 59.1, 50.1 and 77.3 respectively. If one may judge from the other tests, it is the group given the lowest dose which is out of line in this series (Table Ic).

In our fourth experiment a greater spread of doses was chosen, namely 1, 3 and 9 μg., a series nearer the lower end of the curve. The percentages of birds cured were 8.0, 28.0 and 56.0 respectively, again a graded response to graded doses given (Table Id).

Thus it is evident that when the percentage of birds cured is used as the criterion of activity the response of pigeons is graded to the dose given.

The average duration of the cure of these birds was also noted, but many of the birds that had been cured died suddenly without developing retracted neck again. However, the cure was counted as lasting up to the day of death. It may be seen from Table Ia, b, c, d, that the duration of cure of birds given different doses of crystalline vitamin B<sub>1</sub> by mouth is not graded to the dose given. Yet Coward *et al.* [1933] had obtained responses which, when measured as duration of cure, were graded to the dose of vitamin B<sub>1</sub> given by mouth when the vitamin was contained in (a) an acid clay adsorbate, (b) dried yeast, (c) a concentrate, (d) an extract of rice polishings and (e) a soft extract of yeast. Thus our experience with regard to the "duration of cure" criterion for measuring vitamin B<sub>1</sub> has been similar to that of Kinnersley and Peters. With regard to the use of "percentage of birds cured" as criterion we consider we have ample evidence that the response thus measured is graded to the dose of crystalline vitamin B<sub>1</sub> given orally.

*The accuracy obtainable by this method (% of birds cured)*

The examination of the figures obtained in these tests shows that we did not attain a much greater degree of accuracy than that calculated from our figures by the Sub-Committee on the Accuracy of Biological Assays, Pharmacopoeia

Commission [1936]. The accuracy of a determination based on an "all or none" reaction may be calculated from the following equation:

$$\lambda_M^2 = \left( \frac{1}{S(B'n')} + \frac{1}{S(B''n'')} + \frac{(\bar{y}' - \bar{y}'')^2 \sigma_b^2}{b^2} \right) \times \frac{1}{b^2},$$

in which  $\lambda_M$  is the standard deviation of the logarithm of the ratio of the activities,

$n'$ ,  $n''$  are the numbers of animals in the groups given a dose of Standard and test substance respectively,

$B'$ ,  $B''$  are the corresponding "weight factors" which depend on the observed percentage response,

$y'$ ,  $y''$  are the normal equivalent deviations corresponding to the percentage of birds cured,

$b$  is the slope of the curve relating  $y$  to the log of the dose to the base 10,

$\sigma_b$  is the standard deviation of  $b$ .

The values for  $y'$ ,  $y''$  are found directly from tables [Gaddum, 1933; Bliss, 1935; Pearson, 1930 (Table I)]. The values for  $B'$ ,  $B''$  are found from the equation  $B = z^2/pq$ , in which  $z$  is found from Pearson's Table II,  $x$  in this table being  $y$  of the present calculation,  $p$  is the proportion of birds cured and  $q$  the proportion not cured.

It is obvious from the form of the equation that (a) the larger the number of animals used, (b) the larger the values of  $B$  (i.e. the more nearly the number of birds cured approaches 50 %) and (c) the steeper the curve of response, the more accurate will the determination be.

In order to gain a general idea of the accuracy obtainable by using a reasonable number of birds in this test, the Sub-Committee on the Accuracy of Biological Assays (Brit. Pharm. Commission) chose the values  $n' = n'' = 10$ ,  $B' = B'' = 0.5$  (which corresponds to about 79 or 21 % of birds cured in each group),  $y' = y''$  (which makes the last fraction in the bracket of the equation vanish, and  $b = 2.00$  (the average of the 6 values, 1.80, 2.64, 1.50, 1.11, 1.25 and 4.0 already available from 6 different experiments). Thus estimated,  $\lambda_M$  was equal to  $\pm 0.3162$ . By multiplying this by 2.576 and taking the antilogs, the limits of error ( $P = 0.99$ ) were estimated as 15 and 650 %.

The results already described in this paper can be used to gain further information on the accuracy of the pigeon method (% cure of birds). The same conditions of experiment as in the B.P. estimation of the accuracy of the method may be assumed, i.e.  $n' = n'' = 10$  and  $B' = B'' = 0.5$  (79 or 21 % of the birds were cured in the two groups given Standard and test substance respectively). The only difference lies in the slopes of the curves of response. These were calculated as regression lines relating the normal equivalent deviation (determined from tables from the percentages of birds cured) and the log to the base 10 of the dose of crystalline vitamin B<sub>1</sub> given. The 4 values for  $b$  found from the 4 experiments were 2.87, 2.57, 1.85 and 2.95 respectively; the mean, weighted according to the number of birds used in constructing each curve, was 2.53, not greatly different from the mean 2.00 used in the B.P. estimation of the accuracy of the test.  $\lambda_M$  was calculated for each of the 4 experiments as  $\pm 0.2204$ ,  $\pm 0.2458$ ,  $\pm 0.3419$  and  $\pm 0.2145$  respectively. The limits of error ( $P = 0.99$ ) were found to be 27-370, 23-430, 13-760, 28-357, mean 21-466, not very different from the first estimate of 15-650.

The probability that there has been a gain in accuracy in the last 4 experiments is very small indeed. Since the slopes of the curves are the determining factors in these calculations of the limits of error, the value of  $t = \frac{M_1 - M_2}{\sqrt{(\epsilon_1^2 + \epsilon_2^2)}}$  has been determined for the two average slopes (2.00 for the first set of 6 figures, used by the Sub-Committee on the Accuracy of Biological Assays, and 2.53 for the 4 experiments described in this paper).  $t$  was found to be 1.145, which is not large enough to indicate a significant difference between two averages of 6 and 4 observations respectively. Hence, unless some means of obtaining a much steeper curve of response can be discovered, it seems unlikely that the test can be made to give more accurate results than have been already obtained.

Table II. *The accuracy obtainable in pigeon tests in which  $n' = n'' = 10$ ,  $b = 2.00$ ; the percentage of birds cured is the same in both groups (on Standard and test substance respectively) and ranges from 50 to 5 or 95 %*

% birds cured	$\lambda_M$	$\lambda_M \times 2.576$	Limits of error
			$P = 0.99$
50	$\pm 0.28027$	$\pm 0.7220$	19-527
40 or 60	$\pm 0.28362$	$\pm 0.7306$	19-538
30 or 70	$\pm 0.29517$	$\pm 0.7604$	17-576
20 or 80	$\pm 0.31954$	$\pm 0.8230$	15-665
10 or 90	$\pm 0.38228$	$\pm 0.9848$	10-966
5 or 95	$\pm 0.47272$	$\pm 1.2177$	6-1650
0 or 100	$\alpha$	$\alpha$	0- $\alpha$

A calculation has been made of the accuracy obtainable in similar pigeon tests with  $b = 2.00$ ,  $n' = n'' = 10$  and  $B$  calculated from the assumption that both groups had equal percentages of birds cured, ranging from 50 to 5 or 95 %. It is summarized in Table II. It is evident that the accuracy does not vary much when the results lie between 30 and 70 % or even between 20 and 80 %, but beyond those limits the accuracy decreases very rapidly indeed, and, very obviously, no faith whatever could be placed in a result in which the doses of Standard and test substance respectively cured 100 % of birds in both groups.

## SUMMARY

Four experiments have been carried out in which it has been found that the responses of pigeons were graded to the dose of crystalline vitamin B<sub>1</sub> given. The criterion used was the percentage of birds cured of retracted neck; the doses were given orally. These findings confirm and greatly augment the evidence for the statement of Kinnersley & Peters that a relation between effect and size of dose of crystalline vitamin B<sub>1</sub> given orally becomes apparent when the effect is measured by the percentage of birds cured.

When the response was measured by the duration of the cure of those birds which were cured, the response bore no relation to the size of dose of crystalline vitamin B<sub>1</sub> given. This confirms the statement of Kinnersley & Peters that the "day-dose" method of measuring the response of pigeons to doses of crystalline vitamin B<sub>1</sub> is useless.

Conditions for obtaining the greatest accuracy possible in this method are discussed.

The apparently greater degree of accuracy which seemed to be obtainable after an examination of the figures obtained in these experiments, when compared with the original estimate of the accuracy of the pigeon test, was found not to be significant. The pigeon test, even using the percentage of birds cured,

therefore remains the least accurate of those methods of vitamin assay whose accuracy was investigated by the Sub-Committee on the Accuracy of Biological Assays, British Pharmacopoeia Commission.

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# LXXXIII. THE ABSORPTION OF PHOSPHATES FROM THE INTESTINE

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THE absorption of Ca and P from the alimentary tract is believed to be influenced by a variety of factors, such as vitamin D, the acidity of the intestinal tract, the Ca/P ratio in the diet, parathormone etc. A discussion of all these is beyond the scope of this work: a summary of published work may be found in a paper by Nicolaysen [1937, 1]. Most of the work on absorption, however, has been carried out till now by estimating the intake of Ca and P and the output of these elements in urine and faeces. The amount of Ca or P found in the faeces is usually the sum of the quantity left unabsorbed and that excreted into the intestines. Hence, although the "balance" method might yield useful information about the total retention of Ca and P, it will not give a true picture of absorption alone. Recently the isolated loop technique of Verzář [1935] has been used for studying the absorption of Ca and P. Nicolaysen [1937, 2] found that the absorption of potassium phosphate and sodium glycerophosphate from isolated loops proceeded equally well in normal and vitamin D-deficient rats. He also found that the absorption of inorganic phosphate increased with increasing concentration. Laskowski [1937], using similar technique, also found that vitamin D did not influence the absorption of P, but parathormone did. A reference to both these papers will be made later on.

No information is available about the route by which Ca and P enter the general circulation after absorption from the intestines. There are two possible routes, (1) the portal system and (2) the lacteals, lymphatics of the mesentery and the thoracic duct. So far as the absorption of P is concerned no work has been reported dealing with this aspect. As Schmidt & Greenberg [1935] suggest, one may speculatively assume that after absorption P is carried by the portal blood and enters the general circulation via the liver. In a study on the absorption of amino-acids into the capillaries and the lacteals of the villi Bolton & Wright [1937] could find no evidence of a selective activity on the part of either. Whether such selective activity is also absent in the absorption of phosphate could only be shown by further investigation.

The main purpose of the present investigation was therefore to find the route of absorption of P from the intestines. Additional light has been thrown on the influence of concentration and pH on the absorption of P and on the availability of ester P. These latter aspects are discussed first, and the experiments designed to show the route of absorption are described in the latter part of the paper.

## METHODS

The experiments described in the following pages were carried out on normal healthy dogs. The dog selected for any particular experiment was starved for 16-20 hr. before being subjected to ether or chloroform anaesthesia. The



abdomen was opened with a midline incision and the portal vein was exposed by turning over to one side the intestines, including the duodenum. 5–10 ml. blood were then taken from the portal vein by means of a syringe, a known volume of the phosphate solution, previously warmed to 37°, was introduced into the duodenum by means of a hypodermic syringe and the abdomen was closed by adjusting the flaps and holding them in position by means of forceps. At intervals the abdomen was reopened and 5–10 ml. blood were withdrawn from the portal vein. The experiment lasted for 3–4 hr. during which time the dog was kept anaesthetized and warm.

The blood was always delivered into a tube chilled to 0°; it was gently shaken and portions were measured out for analysis. The P analyses were carried out on whole blood as follows.

*Total phosphorus.* 0.5 ml. whole blood was oxidized with a mixture of  $\text{HNO}_3$  and  $\text{H}_2\text{SO}_4$  (3 : 7). The oxidation was completed with perhydrol and the digested mixture heated long enough to drive out the last traces of  $\text{HNO}_3$ . About 2 ml. water were added and the contents boiled and left overnight. The digest was then made to 50 ml. with the addition of distilled water and P was determined in an aliquot portion by the Bell & Doisy [1920] modification of Briggs's method.

*Acid-soluble phosphorus.* 5 ml. whole blood were mixed with 35 ml. water and 10 ml. 20 % trichloroacetic acid. The mixture was well stirred, allowed to stand for 10–15 min. and filtered. Estimations of total and inorganic P were carried out in aliquot portions of the filtrate.

In later experiments where P estimations in lymph were carried out, a weighed quantity of lymph was treated with trichloroacetic acid and water, made up to a known volume and P was estimated as mentioned above.

*Calcium.* In some experiments Ca also was estimated in deproteinized whole blood filtrates. Ca was precipitated as oxalate and titrated with  $N/100 \text{ KMnO}_4$ , the procedure adopted being that described by Wang [1935].

## I. FACTORS WHICH INFLUENCE THE ABSORPTION OF P

### *The effect of anaesthesia on the P of blood*

In order to be certain about the value of the results obtained by the above technique it was necessary to determine the effect of anaesthesia on the P of blood. For this purpose a dog was starved for 16–18 hr. and a sample of blood withdrawn from a peripheral vein in the hind leg. The dog was anaesthetized with chloroform and ether and kept under anaesthesia for 3–4 hr.; at intervals blood was withdrawn from a peripheral vein and analysed. Since the dog was in the post-absorptive stage there would probably be no difference in the P contents of the portal and peripheral blood. Hence one might reasonably assume that the determination of P in blood from a peripheral vein would give results applicable to the whole venous blood of the anaesthetized animal.

Table I. *The effect of ether and chloroform anaesthesia on blood P*

Time	mg./100 ml. whole blood		
	Total P	Acid-soluble P	Inorganic P
Before anaesthesia	47.53	25.15	3.61
0 hr. under anaesthesia	48.03	29.07	4.73
$\frac{1}{2}$ " " "	48.21	29.07	6.02
1 " " "	49.21	29.20	6.66
2 " " "	47.71	26.60	5.25
3 " " "	47.18	26.26	5.21

This and similar experiments showed that the inorganic P of the blood increased appreciably under anaesthesia and simultaneously, but to a much less extent, the total P of the whole blood also increased. In both cases the maximum was reached within 1 hr., after which the values tended to diminish. Such increase in the inorganic phosphate of the blood has been observed previously. Marenzi & Gerschman [1934] found the inorganic phosphate increased during ether anaesthesia. They state that this increase was not due to changes in the organic P compounds of the blood; the excess inorganic P came possibly from sources other than blood. That this is partly true is evident from a slight increase in the total P of the blood (Table I), but it is also possible that a fraction of the increase in inorganic P might be due to the changes in some of the organic P compounds of the blood, e.g. changes which cause a large increase in the soluble fraction.

It will be clear from the experiment quoted above that in any study of absorption on anaesthetized animals based on estimations of concentration in blood of the substance under investigation, it is necessary to bear in mind the changes which result from anaesthesia. The alterations in the concentration of substances in blood due to absorption from the intestines will, under such conditions, be superimposed on the changes due to anaesthesia. Since these latter are seldom identical it has not been possible to make quantitative allowances for them. The rate of uptake of the substance in question by tissues introduces a further difficulty in the way of a quantitative study of absorption by this method. Thus the concentration in blood of a substance being absorbed from the intestines will depend upon at least two factors: (1) the rate of absorption from the intestines and (2) the rate of removal by the tissues. As it is difficult to obtain reliable information at present concerning the second factor a strictly quantitative interpretation of the results is not possible. Nevertheless it is possible to derive certain useful information concerning the various factors which can influence absorption from the intestines. The results of the experiments described in the following pages have been interpreted after bearing in mind these difficulties.

It is necessary to explain the reason why only whole blood was selected for analysis. As a result of the absorption of P from the intestines there should be a rise not only in the inorganic P of the blood, but also in the total P of the whole blood. It will appear from Table I that in anaesthesia an increase in the inorganic P of the blood can take place without a corresponding increase in the total P. In such experiments on absorption one might easily be led to conclude, on the strength of an increase in the inorganic P only, that a given substance has been absorbed; for instance, experiments with sodium phytate described in Table III (C and D) show a marked rise in inorganic P, but no rise in the total P. In those experiments it would not have been possible to arrive at any conclusion unless the total and inorganic P had both been determined. That this procedure is justified is shown by the close correspondence between the increase in inorganic P and the total P observed in the early stages of absorption of inorganic phosphate (Table II).

No attempt has been made to study the partition of the absorbed P between the plasma and the corpuscles. It is possible that the sudden and large influx of inorganic P in the plasma might upset the equilibrium existing between the several P compounds of the red blood cells and the plasma. The conclusion of Buell [1923] that the red blood cells contain no inorganic P has not been confirmed by Halpern [1936], who has shown that at 37° the membrane of the red blood cell is permeable to phosphate ions, the extent and direction of the

exchange of these ions depending upon the metabolic activity of the R.B.C. It is proposed to investigate shortly the effect which the absorption of P has on the distribution of P compounds in blood.

*The absorption of inorganic phosphate*

Aqueous solutions of  $\text{Na}_2\text{HPO}_4$ , adjusted to definite pH by titrating with solutions of  $\text{NaH}_2\text{PO}_4$  and containing known amounts of P, were introduced into the duodenum and blood from the portal vein was analysed at intervals. The details and results of the experiments are given in Table II.

Table II. *The absorption of sodium orthophosphate*

Particulars	Time, hr.	Total P	mg./100 ml. whole blood		Calcium
			Acid-soluble P	Inorganic P	
A. Wt. of dog, 11.0 kg. Phosphate solution injected, 40 ml. containing 933 mg. P at pH 9.4 (23.3 mg. P/ml.)	0 $\frac{1}{2}$ 1 2 3	59.27 70.06 66.74 63.10 63.73	41.21 51.63 44.80 43.14 43.73	7.19 17.64 14.78 14.61 14.80	6.90 5.60 3.60 3.50 4.00
B. Wt. of dog, 8.13 kg. Phosphate solution injected, 40 ml. containing 870 mg. P at pH 7.0 (21.75 mg. P/ml.)	0 $\frac{1}{2}$ 1 2 3	46.46 49.02 51.12 53.99 ---	28.61 31.56 37.83 36.86 41.16	8.61 12.01 14.28 -- 18.17	6.79 6.43 4.64 2.58 --
C. Wt. of dog, 6.07 kg. Phosphate solution injected, 40 ml. containing 492 mg. P at pH 7.0 (12.3 mg. P/ml.)	0 $\frac{1}{2}$ 1 2 3	46.49 49.62 51.86 52.07 52.94	24.40 28.47 27.97 25.10 26.03	6.14 9.48 10.79 11.36 12.33	7.38 5.58 5.40 -- --
D. Wt. of dog, 8.88 kg. Phosphate solution injected, 35 ml. containing 764 mg. P at pH 4.9 (21.8 mg. P/ml.)	0 $\frac{1}{2}$ 1 2 3	48.09 54.76 55.0 56.9 63.8	27.96 36.94 42.41 42.71 46.59	5.00 12.03 14.65 16.49 19.76	-- -- -- -- --

It appears that the rate of absorption of P is influenced by the pH of the solution introduced. In experiments at pH 9.4, 7.0 and 4.9 the concentration of P per ml. of solution injected was very nearly the same. It was found that pH influenced mainly the rate of absorption and not so much its extent. A rise in inorganic P of more than 10 mg./100 ml. blood was obtained in all these experiments; at pH 9.4, however, the peak was obtained within half an hour of the intra-duodenal injection while at lower pH it was reached in about 3 hr. A comparison of experiments B and C showed that at the higher concentration of P the extent of absorption was more than at the lower concentration. Another interesting observation was that in the early stages of absorption there was a close correspondence between the increase in the inorganic P and the total P of the whole blood, but in later stages the increase in inorganic P over the original level was more than the corresponding increase in the total P.

*The absorption of phosphoric esters*

*Sodium glycerophosphate.* Aqueous solutions of sodium glycerophosphate adjusted to definite pH by dilute HCl were introduced into the duodenum and the experiments were carried out as in the case of inorganic phosphate. The absorption of P took place both at pH 7.0 (Table III, Exp. A) and 4.9 (Table III, Exp. B). When these experiments were compared with those on the absorption

of sodium phosphate (Table II, Exps. C and D) at the corresponding  $pH$  values, it was evident that at  $pH$  7.0 the absorptions of sodium phosphate and glycerophosphate proceeded at practically the same rate, but at  $pH$  4.9 the rate of absorption of the latter was slower than that of the orthophosphate. An explanation of this curious behaviour which suggests itself to the authors is that possibly the rate of absorption of glycerophosphate is limited by the rate of liberation of inorganic P from it by enzymic hydrolysis of the ester. Nicolaysen [1937, 2] found that when glycerophosphate was injected into isolated loops of rat's intestines at  $pH$  6.0 only a very small fraction of total P remaining in the loop at the end of 1 hr. was present as inorganic P, while in a similar experiment at  $pH$  8.5 one to two thirds of the remaining P were found as inorganic P. Nicolaysen therefore concluded that glycerophosphate could be absorbed without previous hydrolysis. How far Nicolaysen's experiments lend themselves to this interpretation is not quite clear. It is possible that at  $pH$  8.5, which is near the optimum  $pH$  for the activity of phosphatase, the rate of hydrolysis of glycerophosphate might exceed that of the absorption of the liberated inorganic P and at  $pH$  6.0 this relation might be reversed. This could provide an alternative explanation of Nicolaysen's observations. Laskowski [1937], also using the loop technique, found that at  $pH$  7.2 the absorptions of inorganic phosphate and of glycerophosphate proceeded practically at the same rate. He further found that those esters which were hydrolysed very slowly were also absorbed very slowly, an observation which suggests that liberation of phosphate in inorganic form is necessary before it can be absorbed. Similar conclusions could be drawn from the experiments described in the present communication (Table III). At  $pH$  7.0, at which the phosphatase might be active, hydrolysis and also absorption might have proceeded at a rapid rate but at  $pH$  4.9 the enzyme,

 Table III. *The absorption of phosphoric esters*

Particulars	Time, hr.	Total P	mg./100 ml. whole blood		
			Acid-soluble P	Inorganic P	Calcium
A. Sodium glycerophosphate.	0	48.58	28.04	2.77	6.41
Wt. of dog, 7.63 kg. 40 ml.	$\frac{1}{2}$	—	30.15	4.35	5.49
injected contained 520 mg.	1	52.64	35.72	7.14	4.59
P at $pH$ 7.0 (13.0 mg. P/ml.)	2	51.25	35.09	7.87	3.60
	3	54.59	38.84	8.70	3.51
	4	53.31	33.34	7.29	3.15
B. Sodium glycerophosphate.	0	50.40	35.68	4.70	—
Wt. of dog, 7.57 kg. 30 ml.	$\frac{1}{2}$	56.30	41.35	7.89	—
injected contained 687 mg.	1	57.20	40.82	10.81	—
P at $pH$ 4.9 (22.6 mg. P/ml.)	2	60.67	39.14	10.46	—
	3	58.68	43.60	10.59	—
C. Sodium phytate. Wt. of	0	60.24	30.15	2.63	—
dog, 10.75 kg. 55 ml. in-	$\frac{1}{2}$	60.81	30.46	3.61	—
jected contained 372 mg. P	1	61.55	31.11	4.11	—
at $pH$ 3.8 (6.76 mg. P/ml.)					
D. Sodium phytate. Wt. of	0	59.46	—	5.15	—
dog, 10.83 kg. 35 ml. in-	$\frac{1}{2}$	59.74	38.98	7.94	—
jected contained 334 mg. P	1	58.88	37.82	7.96	—
at $pH$ 5.2 (9.54 mg. P/ml.)	2	58.46	—	9.30	—
	3	59.17	38.05	8.08	—
E. Calcium glycerophosphate.	0	50.69	27.46	3.67	6.30
Wt. of dog, 9.20 kg. 40 ml.	1	51.98	29.12	5.38	6.86
injected contained 340 mg.	2	54.26	30.98	6.74	7.60
P at $pH$ 4.9 (8.5 mg. P/ml.)	3	—	33.63	7.16	7.23
	4	57.11	35.58	7.77	7.23

being considerably less active on account of the unfavourable H ion concentration, might not bring about the hydrolysis rapidly enough to permit the maximum rate of absorption, and hence the concentrations of inorganic and total P in the blood of the dog absorbing glycerophosphate at pH 4.9 would be considerably lower than in a corresponding experiment with inorganic phosphate.

*Sodium phytate.* A solution of the salt of inositolhexaphosphoric acid prepared according to the method described by Patwardhan [1937] was used in concentrations of 6.76 and 9.54 mg. P/ml. In higher concentrations than these the salt would not remain in solution unless acid were added to depress the pH below 3.0. Actually the two experiments were carried out at pH 3.8 and 5.2. There was a rise in the inorganic phosphate of the portal blood, but the elevation of total P in blood was such as could be obtained merely by anaesthesia. Hence it was considered that phytate was not absorbed. This experiment can be compared with that on calcium glycerophosphate (Table III, Exp. E). The solutions of the respective salts contained practically the same concentrations of P. While in the case of calcium glycerophosphate there was a rise of 6.5 mg. in the total P, in the case of phytate the rise was less than 0.5 mg. From these experiments on organic P compounds the conclusion can reasonably be drawn that P must be released from inorganic combination before absorption and that when it is not liberated by hydrolysis for want of suitable conditions it is not absorbed from the intestines, thus confirming Laskowski's conclusions reached by different methods.

#### *The effect of the absorption of phosphate on blood Ca*

Simultaneously with the rise of inorganic P of the blood as a result of anaesthesia there is observed a slight but definite fall of blood Ca. That there is a fall in the blood Ca under the influence of narcotics had been observed by Cloetta *et al.* [1934] in dogs to which were administered paraldehyde, chloral hydrate or "numal", singly or in mixtures. On the other hand Emerson [1928] reported a rise in serum Ca under ether anaesthesia, but he also found that under ether anaesthesia accompanied by a certain amount of asphyxia serum Ca showed a slight fall. Patwardhan & Chitre (unpublished experiments) observed a slight depression of serum Ca level after the administration of urethane; similar results were obtained after giving a mixture of ether and chloroform. Any change in Ca level which results from the influx of phosphates into the blood of an anaesthetized animal will therefore be superimposed on the changes caused by anaesthesia as in the case of phosphates. Fortunately, however, the fall in blood Ca due to anaesthesia is small, never exceeding 1.0 mg./100 ml. serum.

In the experiments in which P was being absorbed from the intestines the amount of blood Ca was found to fall as the concentration of inorganic phosphate in the blood increased (Tables II and III), this fall being larger than that which would be caused by anaesthesia alone. Binger [1917] noticed that intravenous injection of a large dose of sodium phosphate into a dog lowered the blood Ca. That this was not merely due to the dilution of blood involved in the injection of large volumes of sodium phosphate solution was shown by Tisdall [1922], who injected stronger solutions and obtained similar results. That the amount of Ca in blood is lowered also during the absorption of phosphate from the intestines is evident from the experiments quoted in Tables II and III. But when Ca and P were being absorbed simultaneously the effect on blood Ca was found to be different. For such an experiment it was necessary to ensure that Ca and P could be administered together without the formation of insoluble calcium phosphate.

Such a condition could be fulfilled by using calcium glycerophosphate in acid solutions. Hence an aqueous solution of the calcium salt at pH 4.9 was injected into the duodenum and the Ca and P were estimated in the portal blood at intervals. It was observed that Ca was absorbed more rapidly than P. Although the rise in Ca is, at its maximum value, only 1.4 mg. it becomes significant when it is remembered that ordinarily the level would have fallen much below the original. The absorption of P in this experiment proceeded slowly, more slowly than that of Ca; it was probably limited by the extent of hydrolysis. Higher concentrations of calcium glycerophosphate could not be used as even at pH 4.9 the salt precipitated out of solution.

## II. THE ROUTE OF ABSORPTION

The experiments described in the first section of this paper had shown that the absorption of phosphates proceeded by way of the portal circulation. An alternative route lies via the lacteals, lymphatics of the mesentery and the thoracic duct, for the investigation of which the following addition to the technique already described was made. Lymph can be obtained from the thoracic duct or the *cysterna chyli*. The easiest way of obtaining it, however, is that described by Verzár [1936] for use with rabbits. The lymphatics of the intestines of the dog meet in the root of the mesentery and form into large lymphatics which lead to the *cysterna chyli*. When these lymphatics were cut the lymph oozed out fairly rapidly and could be sucked up by a pipette. It was delivered into weighed tubes which were weighed again to find out the quantity of lymph taken for analysis. This was essential since lymph clotted very rapidly, making it difficult to measure the volume accurately. Trichloroacetic acid was added to deproteinize the lymph and P was estimated as already described. Total P estimations were not carried out on lymph. Blood samples taken at intervals from the femoral or carotid artery were also analysed as before. Two typical experiments are given below, one with sodium phosphate and another with sodium glycerophosphate.

Table IV

Particulars	Time, hr.	Total P	Acid-soluble P	Inorganic P
A. Orthophosphate. Wt. of dog, 10.0 kg. 28 ml. at pH 4.9 injected, containing 862 mg. P (30.78 mg. P/ml.)				
	0		Lymph*	
	0.5		7.49	5.71
	1		10.42	8.46
	2		12.62	11.99
	3		14.52	13.51
			13.27	12.30
			Blood†	
	0	45.4	22.65	5.63
	2	52.6	29.27	12.63
	3	52.5	28.41	12.63
B. Glycerophosphate. Wt. of dog, 8.83 kg. 38 ml. at pH 4.9 injected, containing 744 mg. P (19.58 mg. P/ml.)				
	0		Lymph*	
	0.5		6.26	4.20
	1		11.93	8.20
	2		12.31	11.49
	3		12.43	10.44
			11.62	10.05
			Blood†	
	0	46.5	29.77	3.17
	2	54.2	36.98	10.59
	3	54.0	35.92	9.38

\* mg. P/100 g. lymph.

† mg. P/100 ml. whole blood.

From Table IV it will appear that there is a gradual rise in the inorganic and the acid-soluble P of the lymph which runs almost parallel with that in the arterial blood. The experiments described before showed that P is absorbed via the portal circulation; those described now show that it is also absorbed by the lymphatic route. Patwardhan & Chitre (unpublished experiments) have found that Ca is also absorbed in a similar manner.

This conclusion opens up a new field for work. The problem arises as to whether under certain conditions the absorption of P into the capillaries or the lacteals of the villi could be altered in favour of either of these two routes; it remains also to be seen whether those conditions which affect beneficially or adversely the absorption of P from the intestines have any influence on the route of absorption. Further work is in progress to elucidate these and other connected problems.

#### SUMMARY

The absorption of sodium phosphate, glycerophosphate and phytate has been studied by estimations of P in blood and lymph after the intraduodenal injection of the phosphate solutions into anaesthetized dogs. Orthophosphate was absorbed very rapidly at pH 9.4. The rate of absorption diminished at pH 7.0 and pH 4.9. At pH 7.0 the glycerophosphate was absorbed as rapidly as the orthophosphate, but at pH 4.9 the former was absorbed much more slowly than the latter. Sodium phytate was not absorbed at pH 3.8 or 5.2. Blood Ca fell during the absorption of P, but when calcium glycerophosphate was given the blood Ca rose with the blood P.

When sodium orthophosphate or glycerophosphate was injected into the duodenum the concentrations of inorganic and acid-soluble P in blood and lymph increased considerably, showing that P was being absorbed by the capillaries as well as the lacteals of the villi.

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# LXXXIV. THE HOFMANN DEGRADATION OF GLUTAMINE RESIDUES IN GLIADIN

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THE question of the mode of linkage of the ammonia liberated in the hydrolysis of proteins was reviewed by Damodaran [1932] in his paper describing the isolation of asparagine from an enzymic digest of edestin. This work provided the first direct evidence for the long-standing hypothesis that the ammonia so liberated has its origin in the hydrolysis of glutamine and asparagine residues. Shortly after this, Damodaran *et al.* [1932] announced the isolation of glutamine from an enzymic digest of gliadin. In the course of digestion and isolation large losses occurred which were estimated by the method of Chibnall & Westall [1932]; in this way the amount of free glutamine (and, as Melville [1935] has shown, of glutaminyl peptides) present at each stage was determined. The liberation of ammonia in the enzymic digestion of proteins has since been more closely investigated by Damodaran & Ananta-Narayanan [1938] and Damodaran & Narayanan [1938], who have shown that although pepsin and trypsin do not appear to hydrolyse amides set free in the course of digestion, partial hydrolysis of these is brought about by the acid and alkaline conditions necessary for the action of the enzymes.

There is strong evidence, from the work of Damodaran *et al.* [1932] that a much higher proportion of the glutamic acid of gliadin is present in the form of glutamine residues than the amount isolated by them directly as glutamine; the "indirect" method of Chibnall & Westall [1932] showed that at one stage in digestion the "glutamine amide-N" had risen to 44 % of the "total amide-N". But since the enzymic method of isolation is known to involve large losses due both to the incompleteness of enzymic hydrolysis of the protein and to secondary decomposition of glutamine and glutaminyl peptides, with liberation of ammonia, it seems that some technique other than the use of enzymic hydrolysis is necessary in order to allocate more definitely the ammonia liberated in protein hydrolysis.

It would be particularly desirable if asparagine and glutamine residues in protein could, before hydrolysis, be converted into residues stable to prolonged boiling with acid, and of such a character that they could be isolated with ease from an acid hydrolysate.

The treatment which immediately suggests itself is the Hofmann degradation of amides by alkali and bromine to the next lower primary amine; in this degradation glutamine would become  $\alpha\gamma$ -diaminobutyric acid:



and similarly asparagine would become  $\alpha\beta$ -diaminopropionic acid.

An attempt to degrade free asparagine in this manner, and thus to determine the position of its amide group, was made by Van Dam [1897] without success. Karrer and associates more recently carried out the reaction on *N*-acetyl-asparagine [1923] and *N*-acetylglutamine [1926]; in the degradation of the latter Karrer obtained a yield of 18 % of *l*(+)- $\alpha\gamma$ -diaminobutyric acid. Kanewskaja



[1936] applied the Hofmann degradation to a number of benzamido-acid amides, and obtained good yields of the resulting diamines. She stated that where the benzamido group was in the  $\beta$  position to the  $-\text{CONH}_2$  group, but not in other positions, a glyoxalidone derivative resulted, with elimination of the benzoyl residue. This generalizes the discovery of Karrer & Schlosser [1923] that *N*-acetylparagine on treatment with alkali and bromine gives glyoxalidonecarboxylic acid, which on acid hydrolysis yields  $\alpha\beta$ -diaminopropionic acid.

In the present work it was found that, under the conditions used by Kanewskaja, the yield of *l*(+)- $\alpha\gamma$ -diaminobutyric acid from *N*-acetylglutamine was about 50 %. The procedure finally adopted for isolation of the base was to precipitate it, after acid hydrolysis, with phosphotungstic acid. (Fischer [1901] and Karrer *et al.* [1926] report respectively that *dl*- and *l*(+)- $\alpha\gamma$ -diaminobutyric acids give a precipitate with phosphotungstic acid in the cold.) The phosphotungstic acid precipitate was decomposed with baryta, Ba was removed from the resulting solution with excess of  $\text{H}_2\text{SO}_4$ , and the base, on addition of flavianic acid, crystallized out as the sparingly soluble diflavianate.

In view of the favourable yield of degradation product obtainable from *N*-acetylglutamine, it was decided to apply the procedure to a protein, and subsequently to attempt to isolate *l*(+)- $\alpha\gamma$ -diaminobutyric acid from its acid hydrolysate. Wheat gliadin was chosen for its high glutamic acid and low aspartic acid content, and its content of ammonia equivalent to the glutamic acid present. Gliadin also appeared particularly favourable on account of its low content of basic amino-acids [Osborne *et al.* 1915].

It was thought that the phosphotungstic acid precipitate from the acid hydrolysate of gliadin which had been treated with alkaline hypobromite could be decomposed, and arginine and histidine, if present, removed from the filtrate by the silver-baryta precipitation of Kossel, since this procedure was found not to precipitate *l*(+)- $\alpha\gamma$ -diaminobutyric acid. It was later found that the silver-baryta precipitation could be omitted, since it only precipitated a small percentage of the amino-N resulting from the decomposition of the phosphotungstic acid precipitate. In the final procedure, *l*(+)- $\alpha\gamma$ -diaminobutyric acid was isolated directly as the diflavianate after decomposition of the phosphotungstic acid precipitate. It was identified with the product from glutamine.

Under the best conditions found, with correction for the minimum losses occurring in the course of isolation, a yield of base was obtained, corresponding to 14–16.5 % of the glutamic acid resulting from acid hydrolysis of the protein. This figure is based on an assumed glutamic acid figure for gliadin of 43 % [Jones & Wilson, 1928]. The corresponding figure for glutamine isolated by Damodaran *et al.* [1932] is 5.6 % of the glutamic acid.

The figure given here must represent a lower limit for the percentage of glutamic acid present in gliadin as glutamine, but it gives no indication of an upper limit, since when *N*-acetylglutamine was added to the gliadin solution immediately before degradation the *additional l*(+)- $\alpha\gamma$ -diaminobutyric acid recovered corresponded to a yield of only 10–38 % from the added *N*-acetylglutamine. Skraup [1907] reported that treatment of casein with alkaline hypobromite in the cold greatly diminished the amount of amino-acids obtainable on hydrolysis, giving rise, among other products, to fatty acids and aldehydes. He found the yield of lysine and, surprisingly, histidine to be undiminished, although arginine was completely destroyed. He was unable to isolate glutamic acid from the hydrolysate, using the technique of direct precipitation as the hydrochloride. The present work suggests that most of the arginine and histidine present in the gliadin are destroyed by treatment with

hypobromite at 80°. Goldschmidt and associates [1925-33] have studied the action of cold hypobromite on amino-acids, peptides and proteins, and have suggested a mechanism to explain how tri- and tetra-peptides give rise to nitriles, keto-acids and amino-acids. At the higher temperature employed in the present work considerable destruction of amino-acids certainly resulted. The number of possible side-reactions under these conditions is so large that the low yield of diaminobutyric acid from *N*-acetylglutamine when degraded in the presence of protein is not surprising.

In view of this, no estimate can be made of the yield from the glutamine residues of the protein on degradation, but since acetylglutamine alone gives a yield of about 50%, the yield from these is probably less.

An attempt to increase the yield of diaminobutyric acid by using a peptic digest of gliadin which had been acetylated with a view to stabilizing free amino-groups against attack by alkaline hypobromite was unsuccessful; the yield of base isolated after acid hydrolysis was about the same as that from the original protein.

Thus while the work described here provides a new and fairly direct demonstration of the presence of glutamine residues in gliadin, it only suggests a minimum figure for the amount of these groups present, and gives no indication of how the greater part of the ammonia set free in protein hydrolysis is bound, or of the mode of linkage of the glutamine residues.

## EXPERIMENTAL

### *N*-Acetylglutamine

Glutamine, prepared from sugar-beet pulp, was acetylated with NaOH and acetyl chloride in ether [Karrer *et al.* 1926]. It was subsequently found more convenient to use NaOH and acetic anhydride, according to the procedure of du Vigneaud & Meyer [1932] for acetylation of amino-acids. After acetylation, H<sub>2</sub>SO<sub>4</sub> equivalent to the NaOH used was added, and the mixture was evaporated to dryness *in vacuo*. Extraction with alcohol and crystallization of the product followed the directions of Karrer. Yield 1.45 g. (by either method) from 3 g. of glutamine. The product melted at 199° and had  $[\alpha]_D^{25} - 12.25^\circ$  (water,  $l=2$ ,  $c=2.5$ ). Karrer gives M.P. 199°;  $[\alpha]_D^{25} - 12.5^\circ$  (water,  $l=1$ ,  $c=1.8$ ).

### *Hofmann degradation of N*-acetylglutamine

It was found that the use of KOH was inconvenient, since potassium forms a sparingly soluble phosphotungstate, and is thus precipitated with the diaminobutyric acid. This difficulty was overcome by using NaOH throughout. Otherwise the conditions given by Kanewskaia [1936] were observed.

Bromine (0.85 g.) was dissolved slowly in a solution of NaOH (0.95 g.) in water (13.3 ml.) at -5°. *N*-acetylglutamine (1 g.) was dissolved in this, and the mixture immediately heated to 80°. After 3-5 min. no iodine was liberated on treating a drop of the mixture with acidified KI solution. The mixture was then treated with 25 ml. 6*N* H<sub>2</sub>SO<sub>4</sub>, and refluxed until the amino-N (Van Slyke) had reached a constant value (4 hr.). The mixture was then cooled, and diluted to 120 ml. A solution of 20 g. of phosphotungstic acid (B.D.H.) in 60 ml. water was stirred in, and the mixture was kept for 2 hr. at 0° with occasional stirring. The precipitate was filtered off, and decomposed in the usual way with hot aqueous baryta. The filtrate from the decomposition was distilled *in vacuo* for a few minutes to remove ammonia, and amino-N was determined on an aliquot;

55 % of the original *N*-acetylglutamine-N was present in the solution as amino-N. The solution was then made acid to thymol blue with  $\text{H}_2\text{SO}_4$ , filtered through kieselguhr, and the filtrate was concentrated *in vacuo* to a thin syrup, which was treated with a strong solution of flavianic acid. After some hours at  $0^\circ$  the resulting yellow needles were filtered off, and washed with water. Yield 1.95 g. (50 %) 1(+)- $\alpha\gamma$ -diaminobutyric acid diflavianate. The product could readily be recrystallized from hot water. M.P. (decomp.)  $239^\circ$ . (Found: C, 38.0; H, 3.15; N (Dumas), 11.19; S, 8.26 %.  $\text{C}_4\text{H}_{10}\text{O}_2\text{N}_2 \cdot (\text{C}_{10}\text{H}_6\text{O}_8\text{N}_2\text{S})_2$  requires C, 38.6; H, 2.95; N, 11.26; S, 8.57 %. Found:  $\text{NH}_2\text{-N}$  (Van Slyke, 30 min. shaking), 3.73; calc. 3.75 %.)

The compound yielded 96 % of its amino-N in the Van Slyke apparatus in 6 min. at  $18^\circ$ , and 100 % in 30 min. It had a solubility at  $19^\circ$  of 8.4 mg./ml. in water, 6.2 mg./ml. in 5 % flavianic acid, and 5.9 mg./ml. in  $N/10 \text{ H}_2\text{SO}_4$ . For comparison with the solubilities and properties of the flavianates of other organic bases, including ornithine and lysine, cf. Kossel & Gross [1924].

It was found unnecessary in preparing diaminobutyric acid from glutamine to work up the intermediate *N*-acetylglutamine. In the procedure finally adopted the mixture from the acetylation of glutamine with acetic anhydride and NaOH was treated directly with alkaline hypobromite, and the resulting diaminobutyric acid isolated as the diflavianate in the manner described above. Overall yield 40–50 %.

*Salts of 1(+)- $\alpha\gamma$ -diaminobutyric acid with oxalic acid.* Karrer *et al.* [1926] characterized 1(+)- $\alpha\gamma$ -diaminobutyric acid by means of a salt with oxalic acid, which they described as melting with decomposition at  $205^\circ$  and having the composition of 1 mol. base: 0.5 mol. oxalic acid, without water of crystallization. Fischer [1901] characterized the oxalate of *dl*- $\alpha\gamma$ -diaminobutyric acid as decomposing at  $219^\circ$  and having the composition 1 mol. base: 0.5 mol. oxalic acid: 1 mol. water.

In the present work it was found impossible to obtain a salt agreeing in properties with that described by Karrer. In all, three different oxalates were obtained; the salt which resulted depended on the amount of oxalic acid added to the solution of the base. The following salts were prepared and characterized:

Compound	Mol. diaminobutyric acid	Mol. oxalic	Mol. water
A	1	0.5	1.5
B	1	1	0
C	1	1.5	0

Of these C is the least soluble in cold water and the most convenient to prepare, as an excess of oxalic acid may be added without fear of contamination of the product by higher salts.

The diflavianate was dissolved in hot water, and treated with excess baryta (alkaline to thymolphthalein). The precipitate of barium flavianate was filtered off, and washed thoroughly with  $N/3 \text{ Ba(OH)}_2$ . The pale yellow filtrate was acidified to thymol blue with  $\text{H}_2\text{SO}_4$ , stirred with a little charcoal and filtered. Sulphate was removed exactly from the colourless filtrate with baryta, and an amino-N determination on the filtrate from  $\text{BaSO}_4$  showed that 85 % of the original diaminobutyric acid was present in the solution. 0.5 mol. of oxalic acid per mol. of diaminobutyric acid was then added, and the mixture was concentrated until crystallization occurred. This gave crystals of oxalate A, decomp.  $211^\circ$ . If the diaminobutyric acid was crystallized with 1 mol. of oxalic acid, oxalate B was obtained, decomp.  $206^\circ$ . Compound C could be prepared by

crystallizing from water in the presence of an excess of oxalic acid. Decomp. 177°.

On one occasion, in an attempt to prepare oxalate B, rather more than 1 mol. of oxalic acid was added, and the resulting crystalline product decomposed at 195°. On recrystallization the decomposition point fell to 187°, at which value it remained constant on further recrystallization. This product contained on analysis 1 mol. diaminobutyric acid to 1.25 mol. oxalic acid, but appeared on casual examination with a polarizing microscope to consist of two types of crystal. Debye-Scherrer X-ray powder photographs of this product, and of compounds A, B and C were kindly taken by Mr H. Lipson of the Cavendish Laboratory, Cambridge. Cobalt  $K\alpha$  radiation was used, and examination of the photographs showed that compounds A, B and C gave individually characteristic series of lines, while the product in question gave the patterns of B and C superimposed, and must therefore be regarded as a mechanical mixture of these.

Compounds A, B and C could each be recrystallized from water without change in properties. Mixtures of A with B and of B with C had decomposition points intermediate between those of the two components, and less sharply defined.

Table I shows the properties and analyses of the three compounds, and of the oxalate described by Karrer.

Table I. *Oxalates of l(+)-xy-diaminobutyric acid*

Compound	Decomp. C.	[ $\alpha$ ] <sub>D</sub> (water)	C %		H %		N % Kjeldahl found	% Van Slyke found	N % calc.
			Found	Calc.	Found	Calc.			
A	211	+5.7° +6.0° } (c=1.5)	—	—	—	—	14.8 15.1	14.8	14.75
A (anhydr.)	211	—	36.7	36.8	7.07	6.74	16.8 (Dumas)	—	17.2
B	206	—	35.3	34.6	5.92	5.77	13.2 13.7	13.9	13.48
C	177	—	32.7	33.2	5.30	5.14	10.8	11.3	11.1
Karrer's oxalate	205	+7.3° +7.8° } (c=1.2)	37.0	—	6.50	—	17.04 (Dumas)	—	—

Compound A on drying over phosphoric oxide at 100° in *vacuo* lost 13.35% of its weight. (Calc. for 1.5 H<sub>2</sub>O per mol. of base: 14.2%.)

Compounds B and C (air-dry) lost no weight under the same conditions. Karrer *et al.* [1926] reported their oxalate as anhydrous.

#### *Isolation of diaminobutyric acid from gliadin after hypobromite treatment*

Gliadin was prepared from wheat gluten (B.D.H.) by the method of Nolan & Vickery [1937]. The air-dry material contained 7.5% of moisture, and had N 16.2% (Kjeldahl)—corresponding to N 17.5% in the dry protein.

The method of carrying out the degradation of the protein, and isolation of the resulting base described here is that finally adopted as a routine procedure. The effect of varying some of the conditions is described below.

In a typical experiment 1.5 g. of air-dry gliadin were shaken for 2 hr. with 40 ml. *N* NaOH at room temperature. By this time all solid matter had dissolved. The solution was cooled to -5°, and a hypobromite solution, prepared as above from 80 ml. water, 5.6 g. of NaOH and 5 g. of bromine was added. The mixture was then heated rapidly to 80°. After 3 min. practically all the bromine had disappeared (acid KI test), and the almost colourless solution was acidified with 220 ml. HCl (sp. gr. 1.16) and refluxed for 20–24 hr. At the end of this time

the solution was evaporated *in vacuo* until a large quantity of salt had separated. This was filtered off, washed with strong HCl, and the combined filtrate and washings were further concentrated to dryness. The residue was dissolved in 70 ml. water, 4 ml. H<sub>2</sub>SO<sub>4</sub> were added, and a solution of 11 g. of phosphotungstic acid in 35 ml. water was stirred in. The rest of the isolation was carried out in the manner already described. The amino-N present in the solution from the decomposition of the phosphotungstic acid precipitate after removal of ammonia was 8.5% of the total N of the protein used, whereas the corresponding figure for a hydrolysate of untreated gliadin was 4.5%. On acidifying the solution with H<sub>2</sub>SO<sub>4</sub>, filtering off BaSO<sub>4</sub>, and carrying out a silver-baryta precipitation [Kossel, 1928], 60% of the amino-N from the phosphotungstic acid precipitate derived from untreated gliadin was found to be precipitated, whereas the same procedure, using the treated gliadin, precipitated only 15% of the amino-N. An experiment with a solution of diaminobutyric acid oxalate showed that none of the base was precipitated by the silver-baryta procedure.

It was found that the silver-baryta precipitation could be satisfactorily omitted, and the diaminobutyric acid isolated by treatment with excess of flavianic acid in the presence of free H<sub>2</sub>SO<sub>4</sub> at a volume of 20 ml. After 12 hr. at 0° crystallization was complete; the product on drying decomposed at 237°, Yield 250–320 mg. On recrystallization, the decomposition point was 239°, not depressed on admixture with the diflavianate derived from glutamine. (Found: C, 38.2; H, 3.38; N, 11.3; S, 8.39; amino-N ( $\frac{1}{2}$  hr. Van Slyke) 3.70%. Calc. for C<sub>4</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub>·(C<sub>10</sub>H<sub>6</sub>N<sub>2</sub>O<sub>8</sub>S)<sub>2</sub>: C, 38.6; H, 2.95; N, 11.3; S, 8.57; amino-N, 3.75%.)

A rough estimate of the effect on the yield of varying the conditions of degradation was obtained from the amino-N figure of the solution from the decomposition of the phosphotungstic acid precipitate after removal of ammonia. Table II summarizes the results.

Table II

Temp. of treatment 0° C.	Time of heating min.	Vol. hypobromite solution added (% of amount used above)	Amino-N (as % of original total N)
80	5	20	4.7
80	5	65	7.0
80	3	100	8.5
80	5	130	6.0
80	5	160	4.8
80	5	200	4.0
65	12	160	4.5
50	12	160	5.0

The yield from the degradation carried out under optimum conditions as already described, using 1.5 g. of air-dry gliadin, was corrected for losses in isolation of the base, as follows: *l*(+)- $\alpha$ -diaminobutyric acid oxalate corresponding to 593 mg. of the diflavianate was dissolved with 6.5 g. of NaBr and 6 g. of NaCl in 350 ml. 20% HCl, and refluxed for 24 hr. The isolation of the base was then carried out exactly as described above, 416 mg. of diflavianate being obtained. Thus the loss in isolation corresponds to about 180 mg. of diflavianate. Applying this correction, the diaminobutyric acid (expressed as free base) is found to be 68–79 mg. from 1.5 g. of air-dry gliadin. (1.39 g. of dry protein.) Expressed as glutamic acid, this is equivalent to 6.1–7.1% of the dry protein, or, employing the figure of Jones & Wilson [1928] for the glutamic acid content of gliadin as 43%, 14–16.5% of the glutamic acid residues present.

*Isolation of diaminobutyric acid from an acetylated peptic digest*

The same procedure was carried out on an acetylated peptic digest. 1.5 g. of air-dry gliadin were suspended in 54 ml. *N*/20 HCl, and a solution of 80 mg. of a commercial peptic preparation ("Glanoid") in 8 ml. *N*/20 HCl was added, with toluene as antiseptic. The solution had become homogeneous after 2 hr. incubation at 37°. Incubation was continued for 5 days, after which the digest was neutralized with NaOH to phenol red and concentrated *in vacuo* to about 10 ml. It was acetylated with acetic anhydride and NaOH at 0°, using in all 1.7 ml. of acetic anhydride and 20 ml. of 2*N* NaOH. The resulting mixture was then treated with alkaline hypobromite, and after acid hydrolysis the isolation was carried out exactly as described above. Yield of diflavanate was 270 mg., i.e. much the same as that obtained from direct hypobromite treatment of the protein.

When peptic digestion was followed by tryptic digestion and acetylation, the yield of diflavanate on degradation of the resulting mixture was about 50 % of that obtained above.

*Recovery of added N-acetylglutamine*

When 0.10 g. of *N*-acetylglutamine was added to the alkaline solution of 1.5 g. of air-dry gliadin immediately before treatment with hypobromite, 470 mg. of diflavanate were isolated. This is 150 mg. in excess of the best yield obtained from the same amount of protein by itself, and represents a yield of 38 % from the added *N*-acetylglutamine. In another experiment only 10 % recovery was obtained. When it is considered that the yield from added *N*-acetylglutamine is not subject to correction for loss in the course of isolation, it compares unfavourably with the yield obtained by the direct degradation of *N*-acetylglutamine.

C, H, S and N-Dumas determinations were carried out by Dr A. Schoeller, Berlin-Schmargendorf, and Dr G. Weiler, Oxford.

## SUMMARY

1. *N*-Acetylglutamine was subjected to the Hofmann degradation with alkaline hypobromite: a 50 % yield of *l*(+)- $\alpha\gamma$ -diaminobutyric acid was obtained.
2. A method for the isolation of this base by successive precipitation with phosphotungstic and flavianic acids has been developed.
3. The salts of the base with flavianic and oxalic acids have been characterized.
4. After treatment of wheat gliadin with alkaline hypobromite, followed by acid hydrolysis, *l*(+)- $\alpha\gamma$ -diaminobutyric acid was isolated. The best yields, corrected for minimum losses in isolation, were equivalent to 14–16.5 % of the glutamic acid residues of the protein.
5. It is argued that at least this proportion of the glutamic acid of gliadin exists in the intact protein as glutamine residues.

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# LXXXV. THE AMINO-ACID COMPOSITION OF RABBIT MYOSIN

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THE studies of Weber [1933] and others have proved that the intracellular protein of muscle consists of at least three distinct fractions, the globulin myosin, the albumin myogen and globulin X. To these must be added the myoalbumin fraction recently isolated by Smith [1937], although this fraction has not yet been obtained in its native state. According to Smith, the distribution of the intracellular protein of rabbit's muscle is: 67.5 % myosin, 9 % myogen, 22.5 % globulin X and 1 % myoalbumin.

Of these fractions myosin has been studied more thoroughly than the others. It is characterized by an isoelectric point at pH 5.5, has typical globulin solubilities and in presence of salts is extremely susceptible to denaturation. It is distinguished from the other muscle proteins by exhibiting double refraction of flow, a property associated with the presence of long rod-shaped anisotropic particles, and on this account can be certainly identified with the anisotropic substance of the muscle fibril.

Bailey [1937] has summarized the few available data on the chemical constitution of this protein and has made a comparative study of myosins from various sources with regard to their contents of nitrogen, amide-nitrogen, sulphur, methionine, cystine, tyrosine and tryptophan. His results indicate the existence of a fundamental amino-acid plan for the elaboration of myosin in the skeletal muscle of mammals, birds and fish.

In the present analysis the original scheme consisted of three stages:

- (1) The separation of the monoamino-acids from the dicarboxylic acids and the bases by butyl alcohol extraction.
- (2) The isolation of the dicarboxylic acids and the bases in the unextracted aqueous solution.
- (3) The determination of the monoamino-acids by fractional distillation of the esters and subsequent identification of the ester fractions.

This plan had to be revised when it was found that portions of both the bases and dicarboxylic acids were extracted by the butyl alcohol and since these two groups of amino-acids are the more important from the viewpoint of structure they were determined without previous extraction of the hydrolysates with butyl alcohol.

By incorporation of Bailey's [1937] values, 76 % of the N of the protein has been identified, as shown in Table I, while evidence has been obtained suggesting that the remaining 24 % is largely associated with the monoamino-acid group.

The relations between the chemical constitution and the physical properties of myosin will be discussed fully in a separate communication and will only be mentioned briefly here. Astbury & Dickinson [1935] have shown that in the denatured state myosin exhibits the  $\alpha\beta$  transformation, permanent set and supercontraction, phenomena which are associated with the keratin group of



Table I. *Amino-acid analysis of rabbit myosin*

Amino-acid	N % protein-N	Wt. % protein
Glycine	2.1	1.9
Alanine	3.5	3.7
Valine	2.2	3.0
Leucines	7.1	11.1
Proline	0.4	0.5
Phenylalanine	2.0	4.0
Threonine	0.9	1.2
*Methionine	1.9	3.4
*Cystine	0.56	0.77
*Tyrosine	1.6	3.4
*Tryptophan	0.66	0.82
Arginine	13.4	7.0
Histidine	2.8	1.7
Lysine	11.3	9.9
Glutamic acid	12.5	22.1
Aspartic acid	5.6	8.9
Ammonia	7.9	1.6
Total accounted for	76.4	85.0

\* From Bailey [1937].

proteins. In fibre form, therefore, myosin has the same fundamental folded chain structure which is characteristic of the keratins. It seems that myosin in living muscle is in the form of highly organized systems of peptide chains capable of shortening when appropriately stimulated and mainly determining the optical and elastic properties of the tissue. The changes associated with activity must be closely related to the specific chemical configuration of the constituent molecules.

Myosin is found to contain relatively high proportions of glutamic and aspartic acids, lysine and leucine. The dicarboxylic acids, together with the bases, arginine, histidine and lysine, in constituting half the protein molecule, provide a large number of polar side-chains. The cystine plus cysteine content is low, being only 0.77-0.79 % of the protein [Bailey, 1937] and of this 0.67 % is cysteine [Mirsky, 1936]. The presence of a large number of polar side-chains, together with a large number of long non-polar side-chains and an unusually small number of rigid disulphide links, would constitute, according to present theories of protein structure, a relatively open system of polypeptide chains with many centres for the free co-ordination of water molecules.

Of the intracellular proteins myosin has by far the greatest power of hydration, since it possesses an affinity for water of the same order as gelatin. The usual preparation of the native protein precipitated by dilution from salt solution at pH 6.8 centrifuges to a jelly-like mass with a water content of 98-99 %. It can, indeed, form a gel with a protein content of only 0.2 % by weight [von Muralst & Edsall, 1930], while a salt-free preparation after drying in a chilled desiccator for a week still contains 1 g. H<sub>2</sub>O/g. protein [Mirsky, 1937]. Amongst the fibrous proteins its hydration is exceeded only by the mucins, which contain upwards of 30 % of polysaccharide.

The chemical constitution, therefore, fully supports the theories of the relationship between structure and hydration which have been developed by Jordan Lloyd & Phillips [1933]. Furthermore, if an attempt be made to depict the process of muscular contraction as being due to the action of certain products of metabolism on specific groupings present in the peptide chains, it is clear that an open structure, such as that which can be postulated from the chemical data, will

favour the rapid diffusion of these substances and their secondary products into and away from the sites of action. Such ideas emphasize the importance of the present work in the interpretation of muscular contraction.

### EXPERIMENTAL

*Preparation of myosin.* The protein was prepared by extraction of rabbit's muscle with buffered KCl solution and dilution of the filtered solution with 20 vol. distilled water [Edsall, 1930]. This material was redissolved in salt solution and reprecipitated by dilution to give a product free from traces of the other protein fractions of muscle. The various preparations were thoroughly washed with distilled water and finally brought to the state of a fine flour by treatment with alcohol and ether. On drying *in vacuo* they contained 0.3–0.8 % of ash and 2–3 % of moisture. The ash-free N content was 16.8 %.

### Determination of the basic amino-acids

Block's [1934] procedure was followed in detail, using 2–3 g. of protein for each analysis. The values given in Table II are the mean of closely agreeing duplicates and are calculated from the weights of the isolated crystalline derivatives, namely, arginine flavianate, histidine diflavianate and lysine picrate. The experimental values are corrected for the solubilities of arginine silver and lysine phosphotungstate and also for the small overall losses suggested by Tristram (unpublished data).

Table II. *Basic amino-acids of rabbit myosin and myogen*

Amino-acid	Myosin				Myogen			
	Experimental		Corrected*		Experimental		Corrected*	
	Wt. % protein	N % protein-N	Wt. % protein	N % protein-N	Wt. % protein	N % protein-N	Wt. % protein	N % protein-N
Histidine	1.69	2.70	1.74	2.80	2.90	4.67	2.95	4.75
Arginine	6.7	12.7	7.0	13.4	6.0	11.4	6.3	12.1
Lysine	9.5	10.9	9.9	11.3	7.8	8.9	8.0	9.2
Total bases	17.9	26.3	18.6	27.5	16.7	25.0	17.3	26.1

\* See text.

The lysine picrate after recrystallization exploded at 265–266° and the arginine flavianate decomposed at 258–260°. The histidine diflavianate, when first separated, gave a low decomposition point and a high sulphur value. The recrystallized product gave a satisfactory decomposition point at 246° but the sulphur content was still high owing to the presence of free flavianic acid. (Found 8.46 % S. Theory 8.17 % S.) The small amount of material available prohibited further purification of the salt.

Rabbit muscle myogen, kindly provided by Dr E. C. Bate Smith, has also been analysed (Table II). The distributions of bases in the two proteins are very different, the calculated molecular ratios of histidine, arginine and lysine being 2 : 7 : 12 and 2 : 4 : 6 in myosin and myogen respectively.

Precipitation of the basic N under the Hausmann conditions gave values of 29.4 and 29.1 % of the total N of myosin and myogen respectively. These values are as usual 2–3 % higher than those obtained by calculation from the amounts of isolated bases.

*Separation of the monoamino-acids by extraction with butyl alcohol*

Two samples of myosin, weighing 72 g. and 114 g. respectively, were hydrolysed with 10 times their weight of 6N HCl for 30 hr. Aliquots were then withdrawn for analysis (Table III). In each case the ammonia-N was 7.9 % and the

Table III. *Analysis of myosin hydrolysates*

Hydrolysate	Wt. of protein g	Total N g.	Amino-N g.	Amide-N g.
1	72	12.1	9.8	0.96
2	114	19.1	15.4	1.51
Total	186	31.2	25.2	2.47

amino-N (after removal of ammonia) was 81 % of the total N respectively. Bailey's [1937] value for the amide-N (7.2 %) obtained after hydrolysis with 2N HCl for only 3 hr. is a truer estimate of the N originally bound in amide form, since in the present case the action of the relatively strong acid and longer period of hydrolysis has undoubtedly brought about a small amount of deamination.

Each hydrolysate was then freed from the greater part of the excess HCl by repeated alternate concentration *in vacuo* and dilution, the remaining HCl being removed with Ag<sub>2</sub>O and H<sub>2</sub>SO<sub>4</sub> in the usual way. The H<sub>2</sub>SO<sub>4</sub> was then removed as BaSO<sub>4</sub> and in each case the filtrates and washings were collected, concentrated *in vacuo* to incipient crystallization, kept at 0° for 12 hr. and the separated material filtered off (Table IV: fractions 1-1 and 2-1). Additional

Table IV. *Crystalline fractions removed before butyl alcohol extraction*

Hydrolysate	Fraction	Wt. g.	Total N g.	N %
1	1-1	1.41	0.11	7.8
	1-2	2.50	0.25	10.0
	1-3	5.67	0.63	11.1
2	2-1	2.26	0.17	7.5
	2-2	12.7	1.30	10.2

crops of crystalline material were obtained on further concentration of the filtrates (Table IV: fractions 1-2; 1-3; 2-2). All these fractions consisted in very large part of tyrosine and leucine.

The remainder of hydrolysate 1 and one half of that of hydrolysate 2 (referred to henceforth as 2A) were separately extracted *in vacuo* with aqueous saturated butyl alcohol in a continuous extraction apparatus similar to that used by Dakin [1920], whose procedure in the separation and drying of the extracted monoamino-acids was followed throughout. The longer period of extraction required in the case of hydrolysate 2A was due to the lowering of the bath temperature from 40-45° (employed with hydrolysate 1) to 35-40°.

In each case the butyl alcohol mother liquors were concentrated to a small volume *in vacuo* and the material which separated at 0° was centrifuged off before further concentration to remove all solvent and final solution of the residual syrup in water. Traces of brown tar which remained insoluble in water were found to contain only negligible amounts of N. Moreover, the relatively larger amount of N which remained in the butyl alcohol mother liquors of 2A was due to the difficulty of separating the sticky insoluble material which was obtained in the later period of the prolonged extraction.

The summarized results of the butyl alcohol extractions are given in Table V. The loss of total N was due to the escape of ammonia. Considered from the point of view of effectiveness of the method, the figures show that under the different conditions employed the separations were very similar in each case, and as will

Table V. *Fractionation obtained by butyl alcohol extraction*

	Total N		
	g.	As % of total protein-N	Amino-N g.
Hydrolysate 1. Period of extraction 156 hr.			
Butyl alcohol extract			
(1a) Solid amino-acids (22.6 g.)	2.72	22.5	2.53
(1b) Soluble in water	1.04	8.6	0.76
Insoluble in water	0.02	—	—
(1c) Unextracted aqueous residue	6.33	52.0	4.79
Losses (by difference)	0.49	4.1	0.08
Total	10.60		8.16
Hydrolysate 2A. Period of extraction 247 hr.			
Butyl alcohol extract			
(2Aa) Solid amino-acids (15.0 g.)	1.72	18.0	1.68
(2Ab) Soluble in water	1.66	17.4	1.41
Insoluble in water	0.003	—	—
(2Ac) Unextracted aqueous residue	5.07	53.0	3.69
Losses (by difference)	0.35	3.7	0.12
Total	8.80		6.90

be shown later in Table VI, the unextracted residues contained almost identical proportions of dicarboxylic acids, N precipitable by phosphotungstic acid and residual amino-N. The latter, which represents 10 % of the total unextracted N, must be ascribed to monoamino-acids, the extraction of which was therefore incomplete. The total N precipitable by phosphotungstic acid was lower by 3 % than the Hausmann value on the initial hydrolysate, which indicates that part of the basic N, may have been extracted by the butyl alcohol. Moreover, as is shown in a later section, dicarboxylic acids were undoubtedly extracted by the butyl alcohol under the conditions of these experiments. The studies of egg albumin by Calvery [1932] and of cocksfoot protein by Miller (private communication) have revealed similar partial extractions of both these groups of compounds by butyl alcohol, and in view of the efficient fractionation obtained with hydrolysates of casein, gelatin and zein by Dakin [1918; 1920; 1923], it appears that the success of the method depends on the acid-base relationships in the hydrolysate as prepared for extraction.

*Isolation of the dicarboxylic acids after extraction of the monoamino-acids with butyl alcohol*

Fractions 1c and 2Ac were of similar composition for they contained 52 and 53 % of the total protein-N of which 76 and 73 % were amino-N respectively. Each solution was made alkaline to phenolphthalein with baryta, concentrated *in vacuo* to remove ammonia and Ba removed completely with  $\text{H}_2\text{SO}_4$ . The clarified solutions were then concentrated to a small volume *in vacuo*, saturated at 0° with HCl and two crops of glutamic acid hydrochloride removed in each case. At this stage opportunity was taken to compare the efficiencies of the two routine procedures for the separation of the dicarboxylic acids by precipitation of the Ca salts [Foreman, 1914] and of the Ba salts [Dakin, 1920] with alcohol.

The mother liquors from the separation of glutamic acid hydrochloride were freed as far as possible from HCl by concentration *in vacuo*, the residual syrups dissolved in water and saturated with either  $\text{Ba}(\text{OH})_2$  or  $\text{Ca}(\text{OH})_2$  at a concentration of 2.5–3.0 g. N in 100 ml. aqueous solution; 9 vol. 95 % ethyl alcohol were then added slowly with rapid stirring and after 15–18 hr. at room temperature the insoluble salts were separated and washed with alcohol. The salts were re-dissolved in water and freed from Ba or Ca as sulphate or oxalate respectively. The filtrates and washings in each case were collected, concentrated and the copper salts formed by addition of  $\text{Cu}(\text{OH})_2$  at  $100^\circ$ . The hot solutions were filtered and on standing overnight at  $0^\circ$  the filtrates yielded large crops of copper aspartate. These were filtered off and Cu removed from the filtrates as sulphide. The solutions at this stage contained a large proportion of non-amino-N as shown by the following analyses: 1c, 2.19 g. N, 1.8 g. amino-N; 2Ac, 0.82 g. N, 0.72 g. amino-N. Basic N was obviously present and it was removed by precipitation with phosphotungstic acid in HCl solution under the usual conditions [Van Slyke, 1911]. The filtrates from the insoluble phosphotungstate precipitates were freed from phosphotungstic acid in the usual way and resaturated with gaseous HCl to give a further crop of glutamic acid hydrochloride. Following this, from 2Ac in which lime had been employed as initial precipitant, two additional crops of copper aspartate and a final crop of glutamic acid hydrochloride were separated. The remaining unidentified residue contained only 0.16 g. N. Fraction 1c, however, in which baryta had initially been employed, contained at this stage such a high proportion of monoamino-acids that, in view of the relatively clean separation obtained from fraction 2Ac, it was decided to reprecipitate the residual dicarboxylic acids as their Ca salts. Following this further crops of glutamic acid hydrochloride and copper aspartate were isolated, leaving a final unidentified residue containing 0.19 g. N.

The analyses of fractions 1c and 2Ac at this stage are summarized in Table VI and the final distribution of the N in the salts insoluble in alcohol is given in

Table VI. *Analysis of fractions 1c and 2Ac and hydrolysate 2B*

Fraction	1c (Using baryta and lime)		2Ac (Using lime)		2B (Using lime)	
	Total N g.	N as % of protein-N	Total N g.	N as % of protein-N	Total N g.	N as % of protein-N
Glutamic acid hydrochloride	1.1	9.1	0.87	9.1	1.19	12.5
Copper aspartate	0.51	4.2	0.41	4.3	0.53	5.6
Precipitated by phospho- tungstic acid	3.15	26.0	2.44	25.6	3.01	31.6
Residual amino-N	0.71	5.9	0.49	5.14	2.91	30.4
Ammonia	0.47	3.9	0.41	4.3	0.76	7.9
Losses on precipitates, etc.	0.39	3.2	0.45	4.7	0.40	4.2
Total	6.33	52.3	5.07	53.14	8.80	92.2

Table VII. Although equivalent amounts of the dicarboxylic acids were eventually isolated from the two fractions, the greater selectivity of Ca is apparent, for both fractions, after removal of approximately 80 % of the total glutamic acid present before Ca or Ba salt precipitation, were of similar composition and the data show clearly the larger proportions of basic N and of N apparently belonging to monoamino-acids which are precipitated as Ba salts.

Table VII. *Comparison of the N precipitated as Ba and Ca salts by means of alcohol*

(Values are % total protein-N)

	1c (Using baryta)	2Ac (Using lime)	2B (Using lime)	
			1st ppt.	2nd ppt.
Total N in salts insoluble in alcohol	21.1	12.0	26.2	21.0
Containing:				
Total isolated dicarboxylic acid N	6.3	5.9	18.0	18.0
N in insoluble phosphotungstate	10.1	4.4	3.0	0.0
Residual amino-N	4.1	1.7	5.2	3.0

*Isolation of the dicarboxylic acids without previous extraction of the monoamino-acids with butyl alcohol*

The remaining aliquot of the second hydrolysate, referred to henceforth as 2B, contained 8.8 g. N, 6.9 g. amino-N and 0.76 g. ammonia-N. Excess  $\text{Ca}(\text{OH})_2$  was added, free ammonia removed by concentration *in vacuo* and the insoluble Ca salts precipitated by addition of 9 vol. 95 % ethyl alcohol at a concentration of 2.0 g. N in 100 ml. aqueous solution. The mixture was kept for 15–18 hr. at room temperature, the precipitate filtered off, redissolved in water and reprecipitated at a concentration of 5.0 g. N in 100 ml. aqueous solution. The reprecipitated salts contained no non-amino-N and were found later to give no precipitate with phosphotungstic acid. Four crops of glutamic acid hydrochloride and two crops of copper aspartate were subsequently obtained by the usual procedure. The final residue, which appeared to be free from dicarboxylic acids, contained 0.28 g. N, all in the amino form.

The results of the complete fractionation are given in Table VI and the efficiency of the Ca salt precipitation of the dicarboxylic acids is given in Table VII. At no stage in any of the three separate analyses were products resembling the hydroxyglutamic acid of Dakin [1918] encountered. If the values for glutamic and aspartic acids and the N removed in the phosphotungstic precipitates from hydrolysate 2B are taken as maximum values for the protein, it is clear that large proportions of the dicarboxylic acids and N precipitable by phosphotungstic acid were extracted by butyl alcohol.

It appears from the results given in Tables VI and VII that the higher concentration of N precipitable by phosphotungstic acid present in fractions 1c and 2Ac led to serious contamination of the insoluble Ca and Ba salts to the extent of 48 % and 37 % respectively of the total N present therein, whereas the corresponding value for hydrolysate 2B was only 11 % of the total N, an amount which was completely removed on reprecipitation of the Ca salts. The procedure adopted for hydrolysate 2B was very efficient, glutamic acid hydrochloride and copper aspartate having been isolated in a pure state with relative ease to account for 86 % of the total N recovered from the Ca salts.

*Preparation of the ethyl esters of the monoamino-acids*

All monoamino-acid residues from the separation of the dicarboxylic acids and the bases from hydrolysates 1, 2A and 2B, all fractions extracted by butyl alcohol (1a, 1b, 2Aa and 2Ab) and fractions 1–2, 1–3 and 2–2 were collected. The

position at this stage is summarized in Table VIII. Discounting the N removed as ammonia the total losses on the various inorganic precipitates were equal to 6.8 % of the protein-N. The material for esterification, although equivalent to

Table VIII. *Position of analysis before esterification*

Fraction	N g.	Amino-N g.	N % protein-N
Glutamic and aspartic acids	4.60	4.60	14.8
Removed as insoluble phospho- tungstates	8.64	5.60	27.7
Tyrosine fractions 1-1 and 2-1	0.28	0.29	0.9
Ammonia plus total losses on precipitates etc.	4.57	2.48	14.7
Residues from dicarboxylic acid separations	0.35	0.35	1.12
Material for esterification	12.77	11.88	41.0
Original values in hydrolysates	31.2	25.2	100.2

41 % of the protein-N, contained, by calculation, 1.05 g. dicarboxylic acid-N and 1.21 g. of N precipitable by phosphotungstic acid. Deduction of these values leaves 33.7 % of the protein-N which can be ascribed to the monoamino-acids.

The summation of the N of the identified amino-acid constituents of the protein—glutamic and aspartic acids = 18.1 %; histidine, arginine and lysine = 27.5 % and the amide-N = 7.9 %—gives a total of 53.5 % of the protein-N and leaves 46.5 % to be accounted for as monoamino-acids. The difference of 12.8 % between this estimate and the above value of 33.7 % is explained by the losses on the various precipitates (6.8 %); the additional N which was precipitated with the base phosphotungstates (31.6 less 27.5 = 4.1 %); the small unidentified residues from the separation of the dicarboxylic acids (1.12 %), and finally the tyrosine fractions 1-1 and 2-1 (0.87 %). These four factors represent essentially monoamino-acid-N equivalent to 12.9 % of the protein-N and equivalent to 28 % of the estimated total monoamino-acid-N of the protein. This was rather a large handicap with which to commence the most difficult part of the analysis, but since very little was known regarding the monoamino-acid constitution of myosin, it was decided to obtain by fractional distillation of the ethyl esters as much information as possible about the relative proportions of the monoamino-acids present.

The dry finely powdered amino-acid fractions were suspended in absolute alcohol and the mixture saturated with dry gaseous HCl until complete solution of the amino-acids had been obtained. The solution was then refluxed gently for 30 min., during which time a slow current of the gas was passed continuously. After being cooled to room temperature the solution was freed as much as possible from the water of esterification by alternate concentration *in vacuo* and dilution with absolute alcohol. This complete process was repeated twice. The final concentrate of the ethyl ester hydrochlorides was dissolved in absolute alcohol and adjusted to approximately pH 4 by addition of dry alcoholic ammonia as recommended by Foreman [1919] to remove most of the excess HCl. The  $\text{NH}_4\text{Cl}$  which crystallized from solution at 0° was filtered off. Alcohol was removed from the filtrate by alternate concentration *in vacuo* and dilution with dry chloroform, and the final syrupy residue taken up in dry chloroform to permit the preparation of the free esters from their hydrochlorides by treatment with anhydrous  $\text{Ba}(\text{OH})_2$ .

It was found impossible to obtain a Cl-free solution of the esters by simply shaking the chloroform solution in contact with excess  $\text{Ba}(\text{OH})_2$ , and it was necessary to transfer the mixture to a "runner mill".<sup>1</sup>

The finely divided solid residue of material insoluble in chloroform was removed and washed thoroughly with dry chloroform. Ba was removed quantitatively, the amino-acids in solution re-esterified and a second chloroform solution of free esters obtained.

After the first esterification 79 % of the N present was obtained in the form of free esters in chloroform solution. The re-esterification of the Ba residues brought an additional 13 % of the total N into chloroform solution. 7 % of the N remained in the Ba residues from the second esterification and 1.4 % of the N was lost in the removal of Ba from the residues of the first esterification. The necessary data are given in Table IX.

Table IX. *Distribution of N after liberation of esters*

Fraction	N g.	Amino-N g.
Free esters in chloroform solution	11.70	11.13
In Ba residue after removal of Ba	0.89	0.69
Losses on $\text{BaCl}_2$ and $\text{BaSO}_4$ precipitates	0.18	0.08
Total	12.77	11.90

The chloroform solution of the free esters was concentrated at a bath temperature of 30° and pressure of 100 mm. During this and subsequent concentrations, all vapours which escaped condensation were trapped in HCl to ensure that no loss of volatile esters occurred. On addition of anhydrous ether to the residue, a flocculent precipitate formed. Ether was added until no further precipitation took place. The precipitated esters were filtered off and the ethereal solution after standing overnight at 0° in contact with anhydrous  $\text{Na}_2\text{SO}_4$  was filtered and concentrated to 150 ml. at 100 mm. pressure and a bath temperature of 30°. The concentrated solution of the esters was transferred to the fractional distillation apparatus and the bulk of the remaining ether removed at a bath temperature of 40° and a pressure of 60 mm. Traps cooled by liquid air were used to prevent loss of volatile ester and a slow current of  $\text{CO}_2$  was passed continuously through the ester mixture to promote even boiling.

The ester fraction soluble in chloroform but insoluble in ether contained 0.64 g. total N and 0.39 g. amino-N. The ether solution of the esters contained 11.06 g. total N and 10.74 g. amino-N, these values being equivalent to 86 % of the original N for esterification and to 35 % of the total protein-N.

On completion of the distillation, the small amounts of ester in the liquid air traps were added to their respective main fractions and with the exception of the first fraction the distilled esters were immediately hydrolysed in 6-8 vol. of water at boiling point. The resulting solutions of the free amino-acids were concentrated to dryness *in vacuo* for N analysis and subsequent fractional crystallization.

<sup>1</sup> Owing to the difficulty of obtaining easily a small test sample free from suspended  $\text{BaCl}_2$ , the determination of the presence or absence of  $\text{Cl}^-$  in the chloroform solution of the esters has by all published accounts been rather a difficult step in the procedure. A method for determining the completion of the reaction has been devised by Dr J. W. H. Lugg and is worthy of particular mention. A clean sample can be obtained by touching the surface of the chloroform mixture with a narrow strip of filter paper. After a few seconds the wetted strip is withdrawn and the lower part which contains traces of  $\text{BaCl}_2$  is immediately cut away. The upper part wetted by capillary action contains a clean sample of the chloroform solution which can be washed out and tested for  $\text{Cl}^-$ .



Table X. *Fractions obtained after distillation of the esters*

Fraction	Pressure mm.	Bath temp. °C	Vapour temp. °C	Vapour temp. Bulk of fraction °C	Wt. of ester g.	Recovered acids			
						N g.	Amino-N g.	N %	N % protein-N
1 + trap A	10	40-92	20-49	45-46	36.6	1.89	1.95	—	6.1
2	"	92-98	49-70	60-65	8.7	0.89	0.86	13.3	2.9
3 + trap B	10	98-102	70-73	70-71	8.9	0.74	0.69	11.7	2.4
4	5	88-90	57-61	60-61					
5 + trap C	5	90-100	61-63	62-63	18.2	1.69	1.46	11.6	5.4
6	1.5	80-96	40-41	40	9.6	0.65	0.58	11.5	2.1
7	"	96-110	41-48	44	4.9	0.42	0.39	10.8	1.4
8	"	110-113	51-60	54	2.8	0.22	0.22	10.5	0.7
9	"	113-130	60-81	79	5.4	0.47	0.46	11.0	1.5
10	"	130-146	81-85	84	5.8	0.49	0.46	10.4	1.6
11	Distillation residue (Ether phase)				0.80	0.70	0.70	—	2.6
12	Esters soluble in chloroform, insoluble in ether				1.98	1.63	1.63	—	6.4
13	Trapped during removal of chloroform and ether				0.64	0.39	0.39	—	2.1
14	Ba residues from 2nd esterification				0.32	0.27	0.27	—	1.1
	Loss on 1st BaSO <sub>4</sub> precipitate				0.89	0.69	0.69	—	2.8
	Loss in distillation				0.18	0.08	0.08	—	0.6
	Original values				0.50	1.07	1.07	—	1.6
					12.77	11.90	11.90	—	41.3

The details of the distillation and preliminary analysis of the various fractions are presented in Table X.

*Separation and identification of the monoamino-acids*

**Fraction 1.** This fraction was expected to consist mainly of glycine ester and was treated by saturation with gaseous HCl in alcoholic solution; 3.18 g. glycine ester hydrochloride were obtained containing 10.1 % N (theory 10.0 % N). To the mother liquor and washings were added the re-esterified amino-acids of fraction 13 and from the mixed alcohol solution, after concentration and saturation with gaseous HCl at 0°, a second crop of ester hydrochloride was obtained. This material weighed 1.49 g. and the N content of 12.3 % indicated the presence of glycylglycine ester. The fraction was accordingly refluxed in 20 % HCl; glycine was precipitated from the hydrolysate as the nitrilate [Town, 1936]. The precipitated glycine was equivalent to 90 % of the total N of the fraction, which therefore consisted essentially of a mixture of glycine and glycylglycine esters and corresponded to a glycine content of 0.98 g.

No further crop of ester hydrochloride could be separated from the main mother liquor and, after hydrolysis of the esters and removal of HCl, three crops of free acids were obtained from aqueous solution. These crops were apparently mixtures of glycine, alanine and valine and were dealt with individually.

Crop 1 was further fractionated by precipitation with nitranilic acid. 0.45 g. of pure glycine nitrilate was isolated equivalent to 0.18 g. of glycine, and, after removal of reagents, 4.42 g. of alanine (15.8 % N;  $[\alpha]_D^{25}$  in 10 % HCl = +10.4°) were crystallized from solution. The final mother liquor contained N equivalent to only 0.5 % of the protein-N and was neglected.

Crop 2 contained 15.1 % N. A test nitrilate precipitation proved the presence of 0.34 g. of glycine and therefore, by calculation from the N content, the remainder of the fraction consisted of 2.8 g. alanine and 1.08 g. valine.

Without previous removal of the glycine present, the main bulk of the fraction was treated for the separation of alanine and valine by phosphotungstic acid precipitation according to Levene & Van Slyke [1913]: 2.5 g. alanine (16.0 % N;  $[\alpha]_D^{25}$  in 10 % HCl = +10.4°) and 0.22 g. valine (12.1 % N) were isolated. The residue, weighing 1.23 g. and apparently consisting of all the glycine present with a large proportion of valine and traces of alanine, was not investigated further.

Crop 3 weighed 1.73 g. and contained 14.7 % N. By nitranilic acid precipitation the equivalent of 0.37 g. of glycine was isolated as nitrilate, leaving a residue of 1.36 g. (13.6 % N) which remained unidentified. Since no evidence of the presence of any acid other than glycine, alanine and valine was found in the whole of fraction 1, the probable composition of this end-fraction was 0.59 g. alanine and 0.77 g. valine.

From fractions 1 and 13 therefore the amino-acids actually isolated and identified either as free acids or as derivatives were 3.59 g. glycine, 6.92 g. alanine and 0.22 g. valine. The unidentified fractions by calculation from the N values very probably consisted of 0.89 g. alanine and 1.63 g. valine.

**Fraction 2.** By fractional crystallization from aqueous solution no single amino-acid could be separated. The several crops of crystalline material were evidently mixtures of valine and leucine with traces of alanine. By the lead salt separation of Van Slyke & Levene [1909] 2.01 g. of pure valine (N = 11.96 %;  $[\alpha]_D^{25}$  in 20 % HCl = +26.3°) were isolated.

**Fraction 3.** 2.05 g. of leucine (10.7 % N) were separated by crystallization from aqueous solution, and 1.69 g. of valine (12.0 % N;  $[\alpha]_D^{25}$  in 20 % HCl = +25.1°) were isolated by precipitation in acetone solution.

*Fraction 4.* By fractional crystallization from aqueous solution and additional separation of the lead salts 10.7 g. of leucine (10.7 % N;  $[\alpha]_D^{20}$  in 20 % HCl = +20.8°; in water = -3.54°) were isolated. The aqueous mother liquors were collected (0.38 g. N; 0.2 g. amino-N), taken to dryness and the residue extracted with absolute ethyl alcohol to give a fraction insoluble in alcohol (0.15 g. N, all in the amino form), and a fraction soluble in alcohol (0.224 g. N; 0.038 g. amino-N). From the latter 3.02 g. of proline picrate (melting point 150°) equivalent to 1.01 g. of proline were isolated. By difference only 0.062 g. of the non-amino-N in fraction 4 remained unidentified.

At this stage the residual leucine-valine mixtures from fractions 2, 3 and 4 were mixed and partly separated by means of the lead salts. 1.71 g. of valine (11.9 % N;  $[\alpha]_D^{20}$  in 20 % HCl = +25.4°) were isolated, leaving a residue of 1.73 g. which was further treated as described below.

*Fraction 5.* By crystallization from aqueous solution 3.70 g. of leucine (10.7 % N) were isolated. The final mother liquor contained 0.25 g. N. By acetone precipitation 0.6 g. of leucine (10.7 % N) was isolated, leaving a final residue which contained 13.4 % N and which was treated subsequently with the residual fractions of 2, 3 and 4. These were apparently mixtures of leucine, valine, alanine and proline. A small fraction of insoluble lead salts was removed to leave a final solid residue of 7.62 g. containing 1.04 g. N and 0.87 g. amino-N. This mixture was not resolved into its individual components, but assuming that it consisted of proline, valine and alanine its composition can be calculated on amino-N and total N values to be: 1.4 g. proline, 3.36 g. alanine and 2.86 g. valine.

*Fraction 6.* 3.07 g. of leucine (10.7 % N) were isolated by fractional crystallization from aqueous solution. The mother liquors gave a residue containing 12.8 % N which was not identified.

*Fraction 7.* The acids present in this fraction were very soluble in water and had to be recrystallized from increasing concentrations of alcohol. 0.55 g. of leucine (10.7 % N) was isolated in this way, the remaining material being unidentified.

No attempt was made to separate the individual isomerides in the various leucine fractions isolated. The specific rotations in 20 % HCl of the different crops varied between +19.4° and +24.4° at a temperature of 21° and showed evidence of increasing proportions of *d*-isoleucine and/or *nor*leucine with increasing vapour temperature of distillation of the ethyl ester fractions.

*Fractions 8 and 9.* Preliminary titration showed that 7 % and 10 % respectively of the total N of these fractions were dicarboxylic acid-N. Fraction 9 was not investigated further. The mixed acids of fraction 8, however, contained 11.0 % N, were very soluble in water and gave the positive biuret reaction characteristic of threonine and serine, suggesting the presence of the former. The dicarboxylic acids were removed by precipitation of the Ba salts in alcohol, the soluble salts were freed from Ba and the final aqueous solution concentrated to small volume *in vacuo*. Unfortunately at this stage a large aliquot of the solution was lost through breakage but from the remaining aliquot two solid fractions were separated. Fraction A crystallized from 80 % alcohol solution and the mother liquor on being taken to dryness provided a small residue (10.6 % N) which probably consisted of leucine residues.

Fraction A on analysis gave 40.25 % C,<sup>1</sup> 7.73 % H, 11.75 % N and  $[\alpha]_D^{20}$  in 4 % aqueous solution = -20.2°. The theoretical values for *d*-threonine are

<sup>1</sup> All C, H and S determinations were carried out at the Micro-analytical Laboratory, Oxford.

40.31 % C, 7.62 % H, 11.76 % N and  $[\alpha]_D^{25}$  in aqueous solution =  $-28.3^\circ$  [West & Carter, 1937]. The material gave a strong positive biuret reaction, although all the N was present in amino form, and on recrystallization from alcohol gave an increased specific rotation,  $[\alpha]_D^{25}$  in 4 % aqueous solution =  $-23^\circ$  with no significant change in elementary analysis. On heating, the product turned slightly brown at  $220^\circ$  and melted with decomposition at  $240^\circ$  which is  $11-13^\circ$  lower than the melting point of the synthetic *d*-threonine prepared by West & Carter [1937]. As a result of the loss of a large part of the original solution, there was insufficient material for more complete identification, but the elementary composition, positive biuret reaction, specific rotation, melting point and solubilities all indicate that the fraction may be accepted as slightly racemized *d*-threonine. After making allowance for the aliquot lost, the estimated total threonine was equivalent to 1.24 % of the protein and 0.87 % of the protein-N.

*d*-Threonine has been isolated so far from the hydrolysate of only one other protein, namely fibrin, from which it was originally separated and identified to the extent of 0.08 % of the protein by McCoy *et al.* [1935], who estimated that there was possibly a loss of 80-90 % during the course of their procedure for separation of the pure substance. The threonine isolated from myosin was equivalent to 54 % of the mixed acids of fraction 8 and was isolated without difficulty from the dicarboxylic acids and the apparent leucine isomerides present. With previous removal of the dicarboxylic acids and more efficient fractionation of the ethyl esters, a relatively pure threonine fraction could probably be obtained without the large experimental losses involved in the procedure of McCoy and his associates.

*The residue in the distillation flask (fractions 10 and 11).* The dark brown viscous residue was partitioned between ether and water to obtain separation of phenylalanine ester from the remaining material. The ether solution (fraction 10) contained 0.80 g. N and 0.70 g. amino-N, while the ether-soluble aliquot (fraction 11) contained 1.98 g. N and 1.63 g. amino-N. Four crops of phenylalanine hydrochloride with a total weight of 6.93 g. containing 0.48 g. N were separated from fraction 10. The free phenylalanine contained 8.57 % N and gave  $[\alpha]_D^{25}$  in water =  $-16^\circ$ , showing that some racemisation had occurred at the temperature of distillation. The final mother liquor and washings were combined with fraction 11, the Ca salts insoluble in alcohol were removed and the insoluble phosphotungstates separated under the usual conditions. The insoluble Ca salts contained by difference 0.87 g. N and 0.50 g. N was removed in the form of insoluble phosphotungstates. From the remaining solution of monoamino-acids, 1.83 g. of phenylalanine (8.57 % N) were isolated, leaving an unidentified residue which contained 0.77 g. N.

*Fraction 12.* This fraction consisted of the esters which were soluble in chloroform but insoluble in ether. Of the original 0.63 g. N, 0.37 g. was precipitable by phosphotungstic acid, leaving a residue which contained 0.04 g. tyrosine and an unidentified mixture equivalent to 0.8 % of the total protein-N.

*Fraction 14.* The Ba residue from the second esterification was freed from Ba and the N precipitable by phosphotungstic acid removed. Of the original 0.87 g. N in the fraction 0.34 g. was removed by phosphotungstic acid precipitation and 0.91 g. of crude tyrosine containing 0.07 g. N was separated, leaving a final unidentified residue containing 0.42 g. N.

The results of the analyses of the ester fractions are summarized in Table XI. Of the original 12.8 g. N in the material which was esterified, 5.74 g. were associated with the isolated free amino-acids or their derivatives, 0.96 g. was associated with the dicarboxylic acids and 1.21 g. were associated with N precipitable by

Table XI. *Summary of the fractionation of the monoamino-acids*

Ester fractions	Isolated fractions	N identified g.	N unidentified g.
1 + 13	Glycine	0.67	0.42
	Alanine	1.09	
	Valine	0.026	
2, 3, 4	Valine	0.65	1.19
	Leucines	1.36	
	Proline	0.12	
5	Leucines	0.46	0.19
6	Leucines	0.33	0.09
7	Leucines	0.059	0.16
7, 8, 9	Dicarboxylic acids by titration	0.086	0.60
8	Threonine	0.27	
10 + 11	Phenylalanine	0.64	0.77
10 + 11	Basic N (insoluble phosphotungstates)	0.50	
10 + 11	Dicarboxylic acids (insoluble Ca salts)	0.87	
12	Tyrosine (colorimetric)	0.003	0.27
12	Basic N (insoluble phosphotungstates)	0.37	
14	Tyrosine (isolation and colorimetric)	0.065	0.46
14	Basic N (insoluble phosphotungstates)	0.34	
	Losses in esterification	—	0.68
	Total	7.91	4.83

phosphotungstic acid. The remaining 4.83 g. represented incidental losses and the residual fractions of unidentified material in the ester fractions. Although only 18.4 % of the N of the protein was identified as monoamino-acid-N, important information was gained regarding the efficiency of the esterification procedure and the distribution of the amino-acids in the main ester fractions. Attention may be drawn to the following points.

(1) On the basis of the values obtained from hydrolysate 2 B, it was estimated that the dicarboxylic acid-N in the material to be esterified was 1.05 g. The total dicarboxylic acid-N traced by titration in fractions 7, 8 and 9 and by separation of the insoluble calcium salts from fractions 10 and 11 was equal to 0.96 g. This would indicate that the values quoted for the dicarboxylic acids are maximum values for the protein.

(2) The total basic N present in the material for esterification was estimated to be 1.21 g. A total of 1.21 g. N was removed subsequently by precipitation with phosphotungstic acid and this N was distributed in approximately equal proportions amongst the residue from distillation of the esters, the esters which were insoluble in ether and the Ba residue remaining after liberation of the esters from their hydrochlorides.

(3) According to Bailey's [1937] determination the total tyrosine-N is equal to 1.57 % of the protein-N. The free tyrosine which separated during concentration of the Cl-free hydrolysate (see Table IV) contained the equivalent of 0.9 % of the protein-N. The greater part of the remainder, equivalent to 0.22 % of the protein-N, was present in the Ba residues (fraction 14).

(4) The total monoamino-acid-N in the material which was esterified was 10.51 g. ( $12.77 - (1.05 + 1.21)$ ). The N remaining in the Ba residue from the second esterification was 1.07 g. and of this 0.34 g. was subsequently proved to be essentially basic N. The actual monoamino-acid-N which remained insoluble in chloroform was equal to 0.73 g. and therefore 93 % of the monoamino-acid-N was obtained in the form of free esters in chloroform solution. The procedures for esterification and liberation of the esters of the monoamino-acids may be accepted without alteration as highly efficient if account is taken of the known difficulties in the esterification of the hydroxy-acids, tyrosine and serine, which have usually been found in the Ba residue after liberation of the esters.

## SUMMARY

Investigation of the amino-acid composition of myosin from rabbit's muscle has led to the identification of 72 % of the N of the protein, equivalent to 77 % of the protein by weight. By incorporation of the values obtained by Bailey [1937] for tyrosine, tryptophan, methionine and cystine, the total identified material is raised to 76 % of the N of the protein, equivalent to 85 % of the protein by weight.

The basic amino-acids were determined by the Block [1934] procedure, the dicarboxylic acids by separation of the calcium salts and the monoamino-acids by fractional distillation of the ethyl esters.

In the course of the analysis the following observations relating to analytical procedure were made:

(a) Large proportions of both the basic amino-acids and the dicarboxylic acids were extracted from an acid-free protein hydrolysate by butyl alcohol.

(b) Under the conditions of experiment, the calcium salts were more selective than the barium salts for the separation of the dicarboxylic acids.

(c) Foreman's procedure for esterification and liberation of the free esters of the monoamino-acids was effective in obtaining 93 % of the N in the form of free esters in chloroform solution.

The author is indebted to Prof. A. C. Chibnall for his constant interest and advice and to Dr E. C. Bate Smith for his valuable co-operation on many points. Mr R. G. Westall gave technical assistance throughout the investigation.

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# LXXXVI. QUALITATIVE TESTS ON HUMAN ENAMEL PROTEIN

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*(Received 1 April 1939)*

IN a previous communication [Pincus, 1936] it was pointed out that enamel protein, which is of epithelial origin and is usually classed as a keratin, failed to conform to certain of the criteria for keratins. The sodium nitroprusside test for cysteine was applied to enamel protein after hydrolysis with 5 and 25 % KOH, and after hydrolysis with HCl. (Enamel protein is hydrolysed with difficulty.) A negative result was observed on most occasions, but after alkaline hydrolysis a slight positive result was obtained twice. Tiny fragments of wool, hair and hoof, after alkaline hydrolysis, readily gave a well-marked positive result with the sodium nitroprusside test. When boiled with 25 % KOH for 30 min., enamel protein showed no blackening with lead acetate, while wool, hair and hoof all readily showed a marked blackening. The findings of Rosebury [1930] with regard to the difficulty of hydrolysis of enamel protein are thus confirmed, but not the "slight darkening" which he observed with lead acetate.

These observations on enamel protein have now been extended in order to get more definite evidence as to the nature of the protein.

For the tests to be described the enamel protein was collected as before, by decalcifying the enamel with acid and washing the protein; 2 % HCl was used in place of the acetic acid used before. The teeth had not been in formalin.

The material collected in this way contained N, 12.1 % (Weiler), S, 1.2 %, ash, 8.0 % (Schoeller). There was about 2 % Ca and a small amount of fatty material. The protein gave a marked reaction for tyrosine.

Now keratins are usually regarded as proteins very rich in sulphur and particularly in cystine. Human hair contains 16.5 % cystine, sheep wool contains 10.0 % cystine, while silk fibroin which has hitherto been classed with the keratins, contains no cystine [Block & Vickery, 1931].

Block [1937] and Block & Bolling [1939] have subdivided the keratins into the eukeratins (cattle horn is the typical eukeratin), which contain histidine : lysine : arginine in the molecular ratio 1 : 4 : 12, and pseudokeratins (human skin is a typical pseudokeratin) which do not give these ratios and which are less resistant to enzymic hydrolysis than the former.

While it would be desirable to ascertain how enamel protein compares with keratins in respect to its amino-acids, such examination appears at present impracticable because of the small quantities of material available.

A more profitable line of investigation, suggested by Dr M. M. Murray and Dr Neuberger, appeared to be that followed by Goddard & Michaelis [1934]. Two tests were applied: (1) to compare the solubility of enamel protein with that of known keratins, e.g. hair, wool, horn and hoof, in solutions of  $\text{Na}_2\text{S}$ , KCN and

Na thiolacetate; (2) to test the action of trypsin and pepsin on such of these proteins as remained undissolved after they had been treated with the solutions mentioned above.

Hair and wool dissolved in 0.75 *M* Na<sub>2</sub>S, 4 *M* KCN or 1.6 *M* Na thiolacetate in 2 days; horn and hoof took rather longer, while enamel protein was more resistant still. If all these proteins were exposed to the solutions for 24 hr., enamel protein resisted subsequent exposure to trypsin longer than did the other substances. It was previously reported that KCN attacked enamel protein: this observation is now thought to be incorrect. Limitation in the supply of enamel protein necessitates collection over a considerable time, so that such moulds as penicillia may contaminate the tiny collections of protein. It has been found that moulds, which may have attacked enamel protein, are themselves readily attacked in turn by KCN, and in this way the error probably arose. Most of the work has been carried out on pieces of material less than 3 mm. square and very thin; supply of suitable human material is limited.

It has been stated above that wool and hair dissolved in Na<sub>2</sub>S or in Na thiolacetate in a couple of days; if horn, hoof and enamel proteins were treated with Na<sub>2</sub>S or Na thiolacetate for 4 days, only enamel protein resisted subsequent exposure to trypsin for 24 hr.: enamel protein is eventually but slowly attacked by trypsin. Experiments with pepsin gave similar results.

Enamel protein thus appears to differ from characteristic keratins. It has a low sulphur content; it does not give a positive test for cystine. The protein does not dissolve in such reagents as Na<sub>2</sub>S, KCN and Na thiolacetate: after exposure to these reagents it is attacked by trypsin and pepsin at a much slower rate than the typical keratins. Enamel protein is therefore unlike hair, wool, horn and hoof and yet it cannot be classed as one of Block & Vickery's pseudo-keratins, since it is more and not less resistant to enzymic digestion than the true keratins; it appears to be much more resistant than human skin. The results of the tests are set out in Table I, which also provides the summary of results.

#### SUMMARY OF RESULTS

Table I. *Results of tests on keratins and enamel protein*

(A) Lead acetate test			
Wool, hair, horn, hoof	All + ve	Enamel protein	- ve
(B) Sodium nitroprusside test			
Wool, hair, horn, hoof	All + ve	Enamel protein	- ve
(C) Solubility and enzymic hydrolysis			
(a) KCN	KCN	KCN	
(b) Na <sub>2</sub> S	Na <sub>2</sub> S	Na <sub>2</sub> S	
(c) Na thiolacetate	Na thiolacetate	Na thiolacetate	
		Contact with one of the above for 4 days followed by trypsin for 24 hr.	
Contact with one of the above for 2 days	Contact with one of the above for 4 days		
Wool	Dissolves	—	—
Hair	Dissolves	—	—
Horn	Resists	Resists	Dissolves
Hoof	Resists	Resists	Dissolves
Enamel protein	Resists	Resists	Resists

This last series of tests (C) was carried out at 37°.



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# LXXXVII. THE ROLE OF THE AROMATIC AMINO GROUP IN DERANGED PIGMENT METABOLISM

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(Received 30 March 1939)

THE recent widespread use in clinical practice of drugs of the benzenesulphonamide type has been followed by many reports of intoxication. Among the more dangerous conditions described are the cases involving the leucopoietic mechanism and resulting in fatal cases of granulocytopenia [Johnston, 1938]. This peculiar toxic action is shared by probably all the coal tar antipyretics and by drugs of the arsphenamine type. Kracke & Parker [1933] showed that the clinical onset of granulocytopenia was preceded by prolonged or intensive administration of drugs which contained an aromatic amino group. These workers describe cases involving the use of acetanilide, phenacetin or amidopyrine, and others with neoarsphenamine or arsphenamine. They concluded that this toxic action depended on the oxidation reactions of the drugs. This view is supported by Archer & Discombe [1937] and Jennings & Southwell-Sander [1937], both of whom advance evidence that drugs containing an aromatic amino group are also capable of causing methaemoglobinaemia, and of catalysing sulphaemoglobinaemia.

## *Methaemoglobinaemia and sulphaemoglobinaemia*

Equally disquieting are the toxic effects of this class of drugs involving the erythropoietic mechanism. Erysipelas patients treated with aminobenzene-sulphonamide have been reported by Snodgrass & Anderson [1937] to develop cyanosis in 30 % of cases, while of 106 puerperal cases cyanosis was observed in 56 % by Colebrook & Purdie [1937]. This cyanosis has been commonly attributed to methaemoglobin or sulphaemoglobin but it is claimed by Marshall & Walzl [1937] that it is unaccompanied by a decrease in the oxygen carrying capacity of the blood, and this claim is supported by King & Leslie [1938], who suggest that a black oxidation product of sulphanilamide may be the cause of the cyanosis and dark colour of the blood. Nevertheless, methaemoglobin and sulphaemoglobin formation following benzenesulphonamide administration have been reported by Colebrook & Kenny [1936] and confirmed by a number of workers [Discombe, 1937; Kane, 1937; Paton & Eton, 1937, 1, 2]. These apparently contradictory reports are illuminated by earlier work on acetanilide. Evidence has accumulated to show that methaemoglobin can be demonstrated in the blood for several hours after acetanilide has been ingested. It has been demonstrated spectroscopically by Dennig [1900], Cabot [1902], Payne [1935] and Todd [1926] and by reduction in the oxygen carrying capacity of the blood by McEllroy [1919]. At this stage phenols which can be detected in the blood stream are at their highest levels, and cyanosis is deepest, while at a later stage it may be impossible to demonstrate the spectrum of methaemoglobin although cyanosis is still observed [Payne, 1935]. These successive stages were thoroughly elucidated by the experiments of Payne [1935]. The persistent cyanosis observed when these drugs are ingested for long periods has been described variously as livid blue, grey or slaty in colour. Immediately following ingestion the livid cyanosis develops

and at this stage methaemoglobin is undoubtedly a factor, while as the drug is eliminated, slowly with sulphanilamide, phenacetin and amidopyrine, but quickly with phenazone, which is soluble, the livid blue gives way to a muddy-grey colour, which persists long after methaemoglobin and sulphaemoglobin have disappeared. Small quantities of *p*-aminophenol in the tissue have been repeatedly noticed and may account for this condition. If acetanilide is not ingested but introduced parenterally it may not be possible to demonstrate the presence of methaemoglobin even although cyanosis is seen [Young & Wilson, 1926]. Methaemoglobinaemia was observed by Snapper [1922] in dogs which had been given phenacetin, while if uncombined sulphur was given simultaneously, sulphaemoglobin developed instead. The cyanosis ceased on withdrawal of the drug and reappeared when the drug was resumed. Sulphaemoglobinaemia was also reported by Ivens & Vollenhoven [1925] in the case of a woman who took phenacetin for three months. Amidopyrine was reported by Kobert [1926] to produce methaemoglobin. In the many published reports of acute aspirin poisoning methaemoglobin and sulphaemoglobin formation have not been suspected and cyanosis has never been reported [compare Dyke, 1935; Wyllie, 1935; Neale, 1936].

*Ultimate fate of the aromatic amino group in the organism*

From a purely chemical standpoint it is a simple matter to postulate a common degradation type for drugs of this series. Hydrolysis and oxidation of compounds containing the aromatic amino group will usually produce *p*-aminophenol. The ease with which it is produced will depend on the stability of the substituent side-chains or rings. Where the *o*-position is substituted as in arspenamine the conversion will proceed first through the less stable *o*-aminophenol. Where there is no amino group, as in aspirin, oxidation will be slow and the final product is likely to be quinol. Evidence in support of this claim is fragmentary and the only cases clearly proved are those of aniline, dealt with later, and acetanilide. Ellinger [1920] has isolated acetylphenylhydroxylamine from the blood of cats poisoned by acetanilide, while Michel *et al.* [1937] have demonstrated that an enzymic hydrolysis due to the acylase of Abderhalden splits acetanilide to acetic acid and aniline. Liver converts aniline into a brown pigment which they consider to be oxidized *p*-aminophenol. Dakin [1922] has shown that the end-point of acetanilide oxidation *in vivo* and *in vitro* is *p*-aminophenol and this product has been isolated from the urine by Herrick & Irons [1906] and from the blood plasma of dogs by Young & Wilson [1926]. The unchanged drug is excreted as a glycuronate according to Herrick & Irons [1906].

No mention has been made in the literature of the isolation or identification of *p*-aminophenol following the use of amidopyrine or phenazone but degradation products are known.

Jaffé [1901] has isolated the red-coloured compound from the urine of dogs treated with amidopyrine. This compound, rubazonic acid, is a condensation product of two oxidized amidopyrine molecules. Enklewitz [1935] has shown that the reducing action of urine following the administration of the drug is due to the conjugated glycuronate. Meyer [1937] has recently advanced evidence to relate the therapeutic efficiency of the aminobenzenesulphonamide molecule with the oxidation potential of the compound ultimately produced in the tissues. Marshall & Walzl [1937], and Rimington [1938] have both observed the presence of a brown oxidation product following sulphanilamide intoxication. Of aspirin, Sollmann [1936] says it is hydrolysed in the gastrointestinal tract to salicylic acid, the bulk of which is excreted as glycuronates and as sulphates,

while a small amount is oxidized to hydroxysalicylic acid and quinol. This oxidation must be small compared with that of drugs containing the readily oxidized amino group. Of the ultimate fate of *p*-aminophenol itself, Herrick & Irons [1906] say it is excreted by the kidneys as ethereal sulphate.

#### *Methaemoglobin formation*

The mechanism of methaemoglobin formation has been established by Heubner and his collaborators, working with the simple aniline molecule. Although phenylhydroxylamine occurs as an intermediary in the oxidation of aniline, it is not considered to be the actual methaemoglobin-former on account of the ease with which it is irreversibly converted *in vivo* into azoxybenzene [Heubner *et al.* 1923]. When aniline was given to cats by subcutaneous injection of a watery solution, Heubner & Schwedtke [1936] were able to demonstrate that eight times the theoretical molar equivalent of methaemoglobin was formed, at which stage an equilibrium was established when 37 % of the total blood pigment was oxidized. These results justify Heubner's conviction that phenylhydroxylamine was not responsible but that the reduction-oxidation system set up by *p*-aminophenol and *p*-iminoquinone was the effective agent. Iminoquinone would be capable of oxidizing one molecule of haemoglobin into methaemoglobin being itself reduced thereby; it could again be oxidized in the blood when it would be available to start again. This catalytic conversion would proceed until an equilibrium was established. To fill the role in this scheme a drug containing an aromatic amino group with a hydroxyl in the *o*- or *p*-position, or with a potential source of these, is required. It seems possible that a quinol:quinone system would fulfil the same function.

#### *Porphyrin excretion*

The rash, which has been reported by Schwentker & Gelman [1937] in a proportion of cases of sulphanilamide intoxication has been shown to be connected with exposure to sunlight by Newman & Sharlit [1937] and Frank [1937]. The possibility that photosensitization was present was investigated by Brunsting [1937] who reported an increase of urinary porphyrin in two uncontrolled experiments. Porphyrin excretion in fever or liver injury was early observed by Garrod [1900], and is discussed by Gunther [1922] but isolation of the type excreted was not attempted. The normal daily human output of urinary porphyrin has been given as 0–50  $\mu\text{g.}$ , and values outside this range are regarded as abnormal by Schreus & Carrié [1933] and Brugsch [1935]. Fink [1934] characterized the normal urinary porphyrin as coproporphyrin I, which was shown by Bingel [1937] to be a much more active photosensitizing agent than the corresponding series III isomerides. The view expressed by Rimington [1936] that urinary porphyrin excretion furnishes a reliable index of the extent of normal haematopoietic activity has received the support of Dobriner *et al.* [1937]. In a series of experiments with rats, Rimington [1938] showed that with doses of 0.4, 1.39 and 1.5 g. of sulphanilamide per kg. the urinary porphyrin excretion increased to 2.5, 7 and 8 times respectively. These porphyrins were isolated and identified as members of the series III, together with small quantities of series I. Earlier experiments with a drug giving a similar degradation product are those of Schreus [1935] who identified coproporphyrin III in the urine of patients treated with salvarsan.

In view of the close connexion between the drugs of the coal tar antipyretic series and drugs of the benzenesulphonamide series, it was decided to investigate

the effect of the former drugs when administered orally to normal healthy rats maintained on a constant artificial diet. The drugs which were examined were, acetanilide, phenacetin, phenazone, amidopyrine, *p*-aminophenol and aspirin.

#### EXPERIMENTAL WORK ON RATS

Groups of three adult male rats, of the albino strain maintained in this laboratory, and weighing 120–150 g. were used. They were kept in metabolism cages, the grids and sloping bases of which were coated with paraffin wax to avoid contamination with metals. Urine was collected every morning from conical flasks fitted with pear-shaped glass bulbs to avoid any contamination with faeces, and was analysed at 3-day periods. A few drops of toluene were always added to the cleaned flasks to inhibit bacterial growth. The rats were removed to feeding cages for 1 hr. during the forenoon and 1 hr. during the afternoon (a single 2 hr. period during the forenoon on Saturday and Sunday) and were offered unrestricted amounts of synthetic diet. Water was available in the metabolism cages, and in the food cages. The drugs were administered by stomach tube, first thing in the morning, and on an empty stomach and some 2 hr. before offering food. These drugs, finely powdered, were suspended in 2 % acacia mucilage in tap water which was administered as 5 ml. per 100 g. body weight. In the first experiments the dose level for all drugs was fixed at 25 % of the LD 50 which had been previously determined [Brownlee, 1939]. Since the excretion of coproporphyrin in the rat varies from animal to animal a pre-period was included for each group and a control group which was dosed throughout with 2 % acacia mucilage was included.

The details of the extraction of the pigment are reproduced later in this work. Quantitative determinations were made by comparing the intensity of the 550  $m\mu$  absorption band in acid solution with that of a standard. This was a solution of coproporphyrin in 0.5 % HCl containing 10  $\mu$ g. per ml. Comparison was made by diluting the unknown to match the standard, and viewing the solutions with a wave-length spectrometer, where the 550  $m\mu$  band only was observed.

The results presented in Table I show porphyrin excretion for two periods of 3 days without drug, and for three periods of 3 days with drug. For acetanilide the marked rise was immediate and reached 10 times the excretion pre-period. With phenacetin, a rise was not seen until the second 3-day period but the final figure was six times. Phenazone showed a decrease for the first 3-day period and finally rose to four times. This decrease which is noted here and is observed again later is probably due to a temporary depression of haematopoiesis by the drug. In the case of amidopyrine the rise is immediate and the final figure was eight times. With aspirin the first 3-day rise is of the order of 2.5 times, reaching 5 times on the second period. This dose of aspirin proved toxic; two of the animals dying on the first day of the third period, and the other, 2 days later. A post-mortem examination of these animals showed gastric haemorrhage [compare van Bodegom, 1925; Barbour & Dickerson, 1938], while the gross appearance of other organs seemed normal.

A second series of experiments was designed to determine whether the dose levels of the various drugs could be adjusted to give the same degree of porphyrin excretion. The details of the experiments are given in Table II while a summary of the porphyrin excretion only is given in Table III.

It is seen that phenacetin and phenazone are porphyrin-producers of the same order, amidopyrine is twice as potent and acetanilide four times. The case of aspirin has proved difficult to interpret. On the previous experiments it

Table I. *Total urinary porphyrin in  $\mu\text{g.}$  for three rats for 3 days, and erythrocyte counts in millions per  $\mu\text{l.}$  on the first day of each period. Drugs were given once daily from 29 March to 6 April*

Date	Acetanilide 300 mg. per kg.		Phenacetin 300 mg. per kg.		Phenazone 400 mg. per kg.		Amidopyrine 300 mg. per kg.		Aspirin 300 mg. per kg.		Controls 2 % acacia only	
	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$
23 March	8.2	28	6.7	36	10.0	24	8.0	18	8.8	18	8.8	12
	8.0		8.5		8.5		7.2		7.9		10.0	
	8.8		9.2		8.8		9.0		8.3		8.5	
26 March	8.0	24	7.4	32	9.6	20	8.2	18	8.5	28	8.6	18
	9.0		8.6		8.8		7.5		7.5		9.0	
	8.4		9.0		8.8		8.8		8.0		7.8	
29 March	8.5	172	7.2	32	8.2	12	8.2	80	8.6	60	8.6	24
	8.2		8.5		8.2		7.2		7.8		9.4	
	8.2		9.2		8.4		8.8		8.5		8.0	
1 April	7.2	280	6.5	140	8.2	60	6.0	90	3.6	120	8.0	24
	8.4		8.0		8.0		5.4		4.5		9.5	
	7.0		8.2		7.2		6.0		3.5		8.8	
4 April	4.5	330	5.0	200	5.6	72	5.2	140	—	—	8.4	22
	4.5		4.2		5.6		5.0		Dead		9.4	
	5.5		5.5		3.2		3.2		Dead		8.2	
7 April	4.0	—	Dead	—	5.2	—	3.2	—	—	—	8.2	—
	5.2		4.0		5.0		Dead		—		9.2	
	6.0		5.0		Dead		Dead		—		8.6	

Table II. Total urinary porphyrin in  $\mu\text{g.}$  for three rats for 3 days and erythrocyte counts in millions per  $\mu\text{l.}$  on the first day of each period. Two pre-periods of 3 days without drugs are followed by four 3-day periods with drugs. Then follow two 3-day periods when the drugs were withdrawn and finally a period when the drugs were resumed

Date	Acetanilide 75 mg. per kg.		Phenacetin 300 mg. per kg.		Phenazone 400 mg. per kg.		Anidopyrine 150 mg. per kg.		Aspirin 150 mg. per kg.		Controls 2 % acacia only	
	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$	R.B.C. count $\times 10^6$	Porphyrin $\mu\text{g.}$
8 May	8.2 8.0 8.8	15 15 15	7.9 10.0 8.5	15 15 15	8.6 8.2 8.5	32 32 32	8.0 8.8 8.8	20 20 20	8.8 8.5 9.0	8 8 8	8.8 10.0 8.5	20
11 May	8.0 9.0 7.9	12 12 12	8.0 9.8 8.2	15 15 15	8.2 7.8 7.8	28 28 28	8.2 8.5 9.0	22 22 22	7.6 7.8 9.0	6 6 6	9.0 10.0 8.6	24
14 May	8.4 9.2 8.2	18 18 18	8.2 9.8 7.8	24 24 24	7.8 7.8 8.4	Nil Nil Nil	7.6 7.8 9.4	Nil Nil Nil	8.2 8.2 9.4	3 3 3	8.8 10.2 8.2	24
17 May	7.2 6.2 6.0	24 24 24	7.2 6.8 5.8	36 36 36	5.0 5.0 5.2	36 36 36	5.8 5.6 7.0	40 40 40	7.6 8.4 9.5	12 12 12	9.0 9.8 8.0	32
20 May	6.2 5.4 5.6	45 45 45	8.0 5.2 4.4	50 50 50	4.5 5.6 5.2	90 90 90	5.2 5.2 6.2	60 60 60	4.6 5.5 6.2	24 24 24	8.5 9.5 7.2	22
23 May	6.6 5.6 5.8	65 65 65	7.5 5.0 4.2	75 75 75	5.0 5.4 4.2	150 150 150	5.2 5.5 4.0	110 110 110	4.8 5.2 6.4	80 80 80	9.0 9.2 7.2	20
26 May	6.6 5.6 5.4	38 38 38	6.2 5.4 4.0	40 40 40	5.5 4.2 3.8	80 80 80	5.0 5.2 4.5	62 62 62	5.5 5.0 5.5	138 138 138	9.2 9.4 7.8	22
29 May	6.8 5.4 5.2	28 28 28	6.2 5.2 3.0	30 30 30	5.0 5.2 4.8	32 32 32	5.8 5.8 5.2	54 54 54	4.5 5.8 6.0	90 90 90	9.4 9.2 7.5	28
1 June	—	32	—	35	—	60	—	50	—	140	—	22

Table III. *Increase of coproporphyrin excretion in urine from groups of three rats for 3-day periods, following administration of antipyretic drugs*

Porphyrin excretion in multiples of the pre-period								
Drug	Daily dose in mg. per kg.	Drugs given				Drugs withdrawn		Drugs resumed
		1	2	4	5	3	2	
Acetanilide	75	1	2	4	5	3	2	2.5
Phenacetin	300	1.5	2.5	3.5	5	3	2	2
Phenazone	400	nil	1	3	5	2.5	1	2
Amidopyrine	150	nil	2	3	5	3	2.5	2.5
Aspirin	150	0.5	2	4	10	15	11	15
Controls	—	1	1.5	1	1	1	1.5	1

appeared to be as potent as phenacetin or amidopyrine while on this occasion it shows a potency of many times that of amidopyrine. Attempts to repeat these experiments with a dose level of 75 mg. per kg. gave an entirely negative response while doses of 150–300 mg. per kg. gave figures which varied from a faint positive to a figure of 15 times normal. These larger figures were always accompanied by haemoglobinuria and the animals very quickly lost tone and died. Post-mortem examination always showed gastric haemorrhage.

Further experiments with these drugs and with *p*-aminophenol were made on groups of twelve adult rats, with the object of isolating the pigments. The protocols are not reproduced again since the results are similar and confirm those given above. In the case of *p*-aminophenol the dose level of one quarter of the average lethal dose (LD 50 = 600 mg. per kg.) was well tolerated. The excretion of porphyrin during the preperiod of 3 days for four rats was found to be 8  $\mu$ g. There was a decrease for the first 3-day period of dosing and the second and third periods showed a small rise of 1.5 times. For the fourth period a jump to 11 times was recorded, while the fifth and sixth periods both gave 22 times the pre-period.

#### *General observations*

Throughout the experiments a daily cycle of events was observed. Before dosing, the animals were quite playful and appeared normal, and at this stage blood was taken from the tail for the blood counts and for methaemoglobin determinations. After dosing, all the animals receiving drugs were depressed, and in the case of animals receiving aspirin, amidopyrine, phenazone and *p*-aminophenol typical muscular spasms were seen. In all cases except that of aspirin, cyanosis developed but passed off in the course of 6–8 hr. During the course of the experiments a tolerance to these drugs was acquired except in the case of aspirin, where the rats became markedly more irritable. All the animals showed losses in weight but the controls always finished with a good appetite, while rats receiving aspirin, phenacetin and amidopyrine showed little interest in food. At the close of the experiments the animals were killed by a blow on the head and the spleens taken for histological examination.

*Urine.* Urine from rats receiving acetanilide, phenacetin and *p*-aminophenol was dark with a greenish fluorescence. On exposure to air it darkened from the top down and rapidly became black. Rats receiving amidopyrine voided a dark red urine, and those receiving phenazone a light pink; both darkened on exposure to air. By qualitative tests all these urines showed increased amounts of protein, bile salts and urobilin. The urine from rats receiving aspirin was dark and darkened a little on standing, and while excess protein was frequently found, urobilin and bile salts were normal. Increases in the volumes of urine, such as Rimington [1938] observed with sulphanilamide were not seen. Blood from all



animals was examined spectroscopically for methaemoglobin and sulphaemoglobin, by approved methods, but only in the case of one animal treated with acetanilide and one receiving *p*-aminophenol was the characteristic spectrum of methaemoglobin identified. This examination was made 22 hr. after administration of the drug when only persistent methaemoglobinaemia would have been identified.

*Blood picture.* A gradual fall, amounting to a progressive anaemia, was observed in the circulating erythrocytes during the course of these experiments. The counts are recorded in Tables I and II.

*Photosensitization.* Animals treated with amidopyrine and phenazone lost all fur from the back and sides of the body. All the drugs examined were fed to groups of two rats for 9 days. The backs of these animals and of two normal rats were shaved and exposed to the ultraviolet irradiation from a mercury vapour arc at 24 in. for 15 min. on 3 successive days. All the rats receiving drugs developed typical red weals readily distinguishable from the erythema of the controls. The photosensitization was, however, of a mild order.

*Histological examination of spleens.* The spleens of all animals receiving the drugs were enlarged and slaty black in colour. Sections from animals receiving acetanilide, phenacetin, phenazone, amidopyrine and *p*-aminophenol showed a common microscopic picture. The sinuses were engorged with red blood cells, while the cells which lined them showed a marked reticular hyperplasia. The prussian blue reaction showed a quantity of stainable iron but much more conspicuous were large deposits of brownish non-staining pigment in the pulp.

Sections of spleens from animals treated with aspirin showed smaller amounts of stainable iron and of brown non-staining pigment. The sinuses and cells of the pulp presented a grossly abnormal picture being packed solidly with red cells, most of which were abnormal in outline and size.

The sections when examined by ultraviolet fluorescence microscopy showed some orange fluorescence but this was not associated with the characteristic brown pigment deposits.

#### *Extraction of the porphyrins from urine*

Methods of extraction of porphyrins were outlined by Garrod [1894] and were developed by Fischer [1924, 1, 2; 1926] and Fischer & Duesberg [1932] and by Watson [1932]. An excellent scheme for separation and identification of porphyrins was given by Dobriner [1936], and a method more suited to small amounts by Rimington [1936]. The following is the scheme used in this investigation and is drawn from the last two sources.

The urine is strained through glass wool and washings added; 0.1 vol. glacial acetic acid is added and the solution extracted with 1.5 vol. ether by shaking vigorously for 10 min. continuously. The ether phase is separated, and extraction repeated, but this time with 0.05 vol. glacial acetic acid. The ether is separated, and a third extraction made with 1 vol. ether alone, separated and the three ether-soluble porphyrin extracts are mixed. Trouble may be experienced at this stage by formation of emulsions. These are best broken by carefully floating a few ml. of glacial acetic acid on the surface and leaving undisturbed for 30 min. Acetic acid is now washed out by adding 0.5 vol. distilled water and a quantity of saturated potassium acetate equal to one-fifth of the total volume of acetic acid used. This is repeated once, then followed twice by distilled water alone.

The porphyrins are extracted from the ether by repeated vigorous shakings with 5 % HCl in distilled water. Completeness of extraction is controlled by

spectroscopic examination, or more easily, by fluorescence in ultraviolet light. A final extraction with 20 % HCl in distilled water, afterwards diluted, may be essential. The 5 % HCl is neutralized to Congo red by dropwise addition of saturated potassium acetate together with a few drops of acetic acid. The process of driving the porphyrins back and forth between ether and 2 % HCl in distilled water is repeated twice or until a clear ruby solution is obtained. This solution in HCl is shaken once with chloroform and separated to remove blue bile pigments and protoporphyrins. The porphyrins are again transferred to 5 ml. of ether and washed with half this volume of distilled water which is carefully and completely separated with the aid of a small funnel as advised by Rimington. The ether is vigorously extracted with 0.5 ml. quantities of 0.5 % HCl until no band in the green is observed in the acid shakings and in the ether. It is possible to extract completely in 4-5 shakings.

*Isolation and identification of porphyrins as methyl esters*

The accumulated acid shakings are transferred to a small volume of ether and evaporated over a sand bath. To this small residue are added 5 ml. of saturated HCl in dry methyl alcohol, and the mixture rotated, stoppered and left 24 hr. at room temperature. To the 5 ml. of esterification mixture are added 5 ml. of chloroform and 50 ml. of ice-cold water; this is repeated with fresh chloroform to carry down the droplets completely and separated. The chloroform is shaken with 2 %  $\text{Na}_2\text{CO}_3$  until the chloroform solution shows the "alkaline porphyrin" spectrum. This solution is repeatedly washed with small amounts of distilled water, filtered and evaporated to dryness. In all the present cases the material at this stage was amorphous; these yields were washed with hot light petroleum and then taken up in 2 ml. of dried ether. To this were added 2 ml. of dried methyl alcohol and the volume reduced to 0.25 ml. by warming on a sand bath. The porphyrin esters precipitated in typical form after standing; they were washed free from mother liquor with methyl alcohol and allowed to dry at room temperature. Melting point and mixed melting point determinations with the methyl ester of authentic coproporphyrin III kindly supplied by Dr C. Rimington were then made on the six samples obtained. The figures obtained are shown in Table IV.

Table IV. *Melting points of the methyl esters of the isolated coproporphyrins*

Drug	M.P.: Re-M.P. ° C.	Mixed M.P. ° C.
Acetanilide	142 : 172	142-146
Phenacetin	145 : 168	144-145
Phenazone	143 : 170	142-144
Amidopyrine	152 : 170	142-145
Aspirin	146 : 172	143-146
p-Aminophenol	142 : 170	140-144

Thus the excreted porphyrins have been characterized as coproporphyrin III by spectra, characteristic crystal form and by mixed melting point determination. Fischer & Treibs [1926] gave for coproporphyrin I methyl ester M.P. 253°, and for coproporphyrin III methyl ester 142° they noted that the latter first melted at 142° and remelted after cooling at 172°.

### DISCUSSION

The administration of drugs of the coal tar antipyretic group to healthy adult rats results in a marked porphyrinuria. These pigments belong to the series III type, together with smaller amounts of series I, and therefore these experiments

fall into line with those of Rimington [1938] with aminobenzenesulphonamide, and of Schreus [1935] with salvarsan.

The reported intoxications following the use of these drugs are all strikingly similar. Drugs containing the aromatic amino group are able to produce methaemoglobinaemia, while the incidence of granulocytopenia following benzenesulphonamide types must now be classified with the so-called idiosyncrasies of amidopyrine, acetanilide, phenacetin and the arspenamine compounds.

It is possible that *p*-aminophenol is the common degradation product for drugs containing a potential source of this compound and in some cases its presence has been demonstrated. This stage once reached, the oxidation-reduction system suggested by Heubner & Schwedtke [1936] satisfactorily explains the production of methaemoglobin.

Aspirin contains no amino group and is unlikely to give rise to a vigorous oxidation-reduction system even when repeatedly ingested. Moreover, it is not suspected of causing methaemoglobinaemia in clinical use. The most likely solution is that this drug does cause an abnormal degradation of haemoglobin, possibly through methaemoglobin.

Fischer and his school have established that blood and bile pigments are members of the series III isomerides. The theoretical precursor of haemoglobin is actioporphyryn III. That this synthesis may involve the formation of small quantities of coproporphyrin series I as a by-product which is found in the urine, is suggested by Rimington [1936]. If this theory is correct it is clear that increased coproporphyrin I excretion must be associated with an increase, or derangement of normal bone-marrow haematopoiesis. Abnormal excretion of coproporphyrin I is found in congenital porphyrinuria, sulphonal poisoning and in many cases where the normal degradation products of haemoglobin are not removed from the blood stream, as in catarrhal jaundice, obstructive jaundice, haemolytic jaundice and in atrophic cirrhosis of the liver [Dobriner, 1936].

The excretion of the series III isomeride has been shown in pigment cirrhosis and liver tumour [Dobriner, 1936]. Its presence following lead intoxication has been widely quoted, for example by Grotepass [1932], Fischer & Duesberg [1932], and Mertens [1937], and it occurs after salvarsan [Schreus, 1935]. Recently its presence has been noted by Rimington [1938], while the results of the present observations must be added to the list.

It is improbable that normal erythrocyte breakdown would ever give rise to coproporphyrin III, for Lemberg [1935] considers that haemoglobin during its normal degradation to bile pigment is oxidized while iron and protein are still in combination, and thus never passes through a porphyrin stage.

While the precise relationship between methaemoglobin and coproporphyrin III has yet to be experimentally demonstrated, a general hypothesis can be stated. Lemberg [1935] has formulated a series of reactions leading from haemins to "green haemins" and thence to biliverdins. This conversion provides an authentic model for the system responsible for the formation of bile pigment in the body. In this conversion the rupture of the ring system is catalysed by iron which is present in an unoxidized form. It seems probable that where the iron is already oxidized, as in methaemoglobin, it cannot play its part in this conversion. Degradation of haemoglobin through methaemoglobin therefore results in coproporphyrin III and not bilirubin.

During the course of the experiments described, an increased breakdown of erythrocytes was taking place as shown by the progressive red cell anaemia and

the abnormal urobilin output. It is possible that the increased coproporphyrin I output implied by the photosensitization of the animals was directly due to haematopoiesis, stimulated by the increased degradation of haemoglobin.

#### SUMMARY

1. Acetanilide, phenacetin, phenazone, amidopyrine, aspirin and *p*-aminophenol have been administered by stomach tube to normal healthy rats in daily doses which corresponded to 25 % of the average lethal dose. The animals developed a porphyrinuria which in some cases reached values equivalent to twelve times the normal. During the period of dosing the animals lost weight and developed a progressive red cell anaemia. A mild degree of photosensitization was observed in all cases.

2. Equivalent doses of phenacetin and phenazone produce the same degree of porphyrinuria; amidopyrine and aspirin are twice as potent, and acetanilide four times, while *p*-aminophenol is more potent than acetanilide. These effects run approximately parallel with the acute toxicities of the drugs.

3. Animals taken for histological examination at the close of the experiments showed grossly abnormal spleens. These were enlarged and slaty-black in colour. The sinuses were much engorged and quantities of stainable iron were seen. Conspicuous were large deposits of brownish, non-staining pigment in the pulp.

4. A marked degree of blood-cell destruction is caused by these drugs as shown by the progressive anaemia and the abnormal urobilin output, but a more fundamental disturbance in blood-pigment metabolism is postulated to account for the progressive train of events which is described.

5. The urinary porphyrins have been isolated and identified as coproporphyrin III in each case. Smaller amounts of coproporphyrin I were also present. The significance of these findings is discussed and attention is drawn to the common chemical relationship of drugs known to cause coproporphyrin III excretion.

6. Evidence to support the claim that *p*-aminophenol is the common active degradation product for drugs containing a phenylamine group is reviewed, and where the amino group is absent the view is advanced that quinol is the probable degradation product.

7. Support is given to the claim that the oxidation-reduction systems set up by aminophenol or quinol are responsible for the oxidation of haemoglobin to methaemoglobin.

8. The hypothesis is advanced that where haemoglobin is oxidized to methaemoglobin the normal conversion into bilirubin cannot occur but is replaced by degradation to coproporphyrin III.

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# LXXXVIII. METABOLISM OF STEROIDS

## I. THE ISOLATION OF 7-HYDROXYCHOLESTEROL AND OF THE "HEPATOLS" FROM OX LIVER

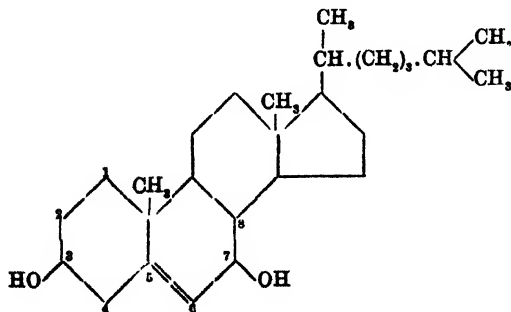
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THE elucidation of the stages in the metabolism of the steroids clearly depends to a large extent upon the isolation and characterization of the intermediates in the processes involved. Many of the substances obtained by Reichstein, Wintersteiner, and Kendall and their colleagues in their work on the adrenal cortex must represent such intermediates; and comprehensive studies such as those of Marker *et al.* on steroid excretion products furnish essential data. Apart altogether from the search for physiologically active compounds, these experiments, together with a large number of more isolated observations [e.g. Marrian, 1929; Haslewood *et al.* 1934; Butenandt & Dannenbaum, 1937; Butler & Marrian, 1938; Burrows *et al.* 1937; Butenandt *et al.* 1937; Callow & Callow, 1938; Beall, 1938] emphasize the need for a complete examination of every type of animal tissue, with the object of the identification of all steroids and related substances present. With this end in view a study of animal liver is being made.

The work here described is the preliminary investigation of a large batch of ox liver. From the unsaponifiable portion of part of this material there has been isolated, as the pure dibenzoate,  $\alpha$ -7-hydroxycholesterol (I). This compound has not been previously found in nature. Its dibenzoate, obtained after Ponndorf reduction of 7-ketocholesterol acetate [Windaus *et al.* 1935], is a well-known intermediate in the laboratory preparation of 7-dehydrocholesterol (provitamin D<sub>2</sub>) which has itself been detected in natural sources [Windaus & Bock, 1937; Bock & Wetter, 1938].  $\beta$ -7-Hydroxycholesterol was obtained by Barr *et al.* [1936] by direct oxidation of the hydrogen phthalate of cholesterol. Possibly the present work will have a bearing on the identification of the "oxycholesterol" repeatedly stated by Lifschütz [1906-9; 1913; 1914; 1921] to be present in animal tissues [cf. Rosenheim & Starling, 1937].  $\alpha$ -7-Hydroxycholesterol gives the colour reactions mentioned by Lifschütz [1914] as characteristic of "oxycholesterol". Its isolation from other animal sources is being attempted.



I

From the same liver fraction there has been obtained also a mixture containing at least two crystalline high-melting alcohols, the "hepatols". These compounds are digitonin-precipitable and can be partly acetylated. They have not yet been completely purified and characterized, but appear to be  $C_{21}$  steroids similar to some of the adrenal cortex substances. There seems little doubt that ox liver will yield other products of this type.

After suitable partition between solvents, 7-hydroxycholesterol and the "hepatols" were first obtained as the digitonides. Whilst the digitonide of the former sterol was readily decomposed by the pyridine method [Schoenheimer & Dam, 1933], treatment with boiling xylene was required for decomposition of the "hepatol" complex. The antimony trichloride reaction described by Barr *et al.* [1936] was of great value in the isolation of the hydroxycholesterol. A method used for the separation of "hepatols" from cholesterol was digitonin precipitation after treatment with excess of bromine, by which means Schoenheimer *et al.* [1930] separated dihydrocholesterol from gall-stone cholesterol.

#### EXPERIMENTAL

Optical rotations were carried out in a 0.5 dm. Fischer micro-tube. All melting points are uncorrected. Analyses were micro-analyses by Dr A. Schoeller.

*Preparation of starting material.* The starting material (supplied by Messrs Boots Ltd.) was dried liver marc, which is the residue left after liver has been extracted with 50 % alcohol, as described in the *British Pharmacopoeia* [1932], p. 171.

*Extraction of marc.* Powdered marc (1 kg.) was extracted with ether in a Soxhlet apparatus until the fresh extract was colourless. Evaporation of the ether gave an oily residue (approx. 200 g.).

*Saponification.* 500 g. of the above ether-soluble product in alcohol (500 ml.) were refluxed for 2 hr. with a solution of KOH (150 g.) in water (500 ml.). The dark solution was diluted to about 5 l. and continuously extracted with ether in a liquid extractor until the fresh extract was colourless. Evaporation of the washed ether gave a crystalline orange-coloured residue (*ca.* 50 g.). This was dissolved in hot methyl alcohol (*ca.* 1200 ml.) and the mixture kept at 0° for 24 hr. The crystalline precipitate was filtered off and washed with cold methyl alcohol, and the filtrate and washings evaporated to give a reddish brown gum (10–12 g.) which was *fraction A*.

#### *$\alpha$ -7-Hydroxycholesterol dibenzoate*

20 g. of *fraction A* were mixed with 250 ml. of 90 % (by vol.) methyl alcohol, and the mixture extracted 6 times with light petroleum (*ca.* 1 l. in all, of B.P. 40–60°). Evaporation of the petroleum gave *fraction B* (18 g.). The alcohol was evaporated and the residue (1 g.), in 70 % (by vol.) ethyl alcohol, extracted 4 times with benzene. The benzene was evaporated, and the residue (1 g.) in 90 % alcohol (20 ml.) treated with a solution of digitonin (1 g.) in 90 % alcohol. Next day the mixture was filtered and the precipitate collected, washed with 90 % alcohol and ether and dried. The product (0.7 g.) was dissolved in dry pyridine (10 ml.) and the solution treated with excess of ether and filtered. The filtrate, washed with dil. HCl and water, was evaporated and the residue washed with light petroleum. A solution of the precipitate (0.2 g.) in dry pyridine (1 ml.) with benzoyl chloride (0.4 ml.) was kept 24 hr. at 0°. The diluted mixture was then ether-extracted and the washed ether evaporated. The residue crystallized from methyl alcohol with a little light petroleum; giving white needles (0.1 g.). After recrystallization from the same solvents the dibenzoate formed white needles, which, alone or mixed with authentic  $\alpha$ -7-hydroxycholesterol dibenzoate

(M.P. 171–172°,  $[\alpha]_D^{25} = +96^\circ$ , in chloroform) had M.P. 172.5–173.5°  $[\alpha]_D^{25} = +95^\circ$  ( $c=0.544$  in chloroform). (Found: C, 80.3; H, 8.85 %.  $C_{41}H_{54}O_4$  requires C, 80.6; H, 8.9 %.) The dibenzoate, the digitonide and the crude 7-hydroxycholesterol each gave an intense blue colour with antimony trichloride in chloroform. Crude 7-hydroxycholesterol gave an intense blue-violet colour with acetic acid and conc.  $H_2SO_4$  (Lifschütz reaction). By no means all the 7-hydroxycholesterol was separated from fraction A by the above method. In experiments with alumina column adsorption followed by benzene washing of the column, 7-hydroxycholesterol was detected only in the upper third of the column.

### *Isolation of the "hepatols"*

A solution of 20 g. of fraction B in approximately 200 ml. of light petroleum (B.P. 40–60°) was extracted 8 times with 90 % methyl alcohol (1 l. in all). The alcohol was washed twice with small portions of light petroleum, evaporated, diluted and ether-extracted. Evaporation of the washed extract gave a gummy residue (ca. 5 g.), which was dissolved in 80 % (by vol.) alcohol and treated with a solution of digitonin (8 g.) in 80 % alcohol. Excess of bromine, dissolved in 80 % alcohol, was now added, when part of the precipitate dissolved. The mixture (ca. 500 ml.) was kept overnight and then filtered. The precipitate, washed with 80 % alcohol and ether, was dried and the product (4 g.) powdered and refluxed for 1½ hr. with purified xylene (25 ml.). The cooled mixture, diluted with ether, was filtered and the residue after evaporation of the filtrate kept for 16 hr. at 0° with a mixture of 10 ml. each of ether and light petroleum. The insoluble material was collected, washed with ice-cold ether/light petroleum, and the white solid (0.6 g.) sublimed at 190–230°/0.05 mm.

*Partial purification of "hepatols"*. The "hepatol" sublimate in a little alcohol was heavily diluted with light petroleum and the solution kept at 0° overnight. White needles (0.2 g.) having M.P. 277–279° were obtained. The compound gave a yellow-orange colour in the Liebermann-Burchard reaction, and was precipitable by digitonin from 80 % alcoholic solution. (Found (a) sample dried *in vacuo* at room temperature C, 71.2; H, 9.9 %; (b) sample dried at 100° *in vacuo* C, 71.8; H, 9.7 %.  $C_{21}H_{36}O_3$ ,  $H_2O$  requires C, 71.2; H, 10.8 %.) A sample was acetylated with pyridine (1 ml.) and acetic anhydride (1 ml.) at 100° for 15 min. The product, precipitated with water, was collected and recrystallized twice from methyl alcohol; from which it formed white needles, M.P. 229–231°. (Found: C, 71.2; H, 9.2 %.  $C_{21}H_{34}O$ ,  $(OCOCH_3)_2$  requires C, 71.4; H, 9.6 %.) On hydrolysis, this product gave white needles, M.P. 284–285°. A sample of "hepatol" digitonide from the brominated mother liquors gave, on decomposition, sublimation and crystallization by the above procedure, followed by a second sublimation, white crystals, M.P. 266°. (Found C, 67.6; H, 9.0 %.)

### SUMMARY

Examination of the unsaponifiable fraction of ox-liver residue (after 50 % alcoholic extraction) has resulted in the isolation, for the first time from a natural source, of the following compounds:

- (1)  $\alpha$ -7-Hydroxycholesterol, characterized as the dibenzoate.
- (2) At least two (partially purified) digitonin-precipitable alcohols, the "hepatols", M.P. ca. 285 and 265°.

The author wishes to express his gratitude to Messrs Boots Pure Drug Co. for the gift of liver marc and to Dr A. S. Parkes of the National Institute for Medical Research, who arranged for the supply of this material.



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# LXXXIX. THE HEMICELLULOSES OF THE WOOD OF ENGLISH OAK

## IV. THE STRUCTURE OF HEMICELLULOSE A

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PREVIOUS work [O'Dwyer, 1934] has shown that the hemicellulose A of oak wood is a mixed polysaccharide containing anhydroxylose and uronic anhydride units and that the sapwood product differs from that obtained from heartwood in that it gives with iodine the blue colour which is characteristic of starch. It has since been shown [O'Dwyer, 1937] that the blue colour reaction given by the sapwood product is accounted for by the presence of anhydroglucose units in the molecular structure and that glucose can be obtained therefrom quantitatively on hydrolysis with takadiastase under controlled conditions. The present communication records a further stage in the fractionation of hemicellulose A isolated from both sapwood and heartwood.

### EXPERIMENTAL

#### (1) *Further fractionation of hemicellulose A by takadiastase*

A series of hydrolyses by means of takadiastase at 38–40° and pH 4.5 [O'Dwyer, 1937] was carried out (a) on hemicellulose A from sapwood after previous removal of its inherent anhydroglucose residues and (b) on hemicellulose A from heartwood after previous digestion with water at 100° for 24 hr. [O'Dwyer, 1934]. Both starting materials showed  $[\alpha]_D^{20} = -97.5^\circ$  (ash-free) in 2 % NaOH ( $c=0.4$ ) and they did not reduce Fehling's solution. 2.5 g. samples were used as before, and the products of hydrolysis were examined after intervals of 46–210 hr. The percentage weight of hemicellulose dissolved at each stage is shown in Table I. The unhydrolysed residue in each case gave a specific rotation of  $-97.5^\circ$ . It was observed that the efficiency of the takadiastase became considerably impaired after 95 hr. at 38–40° when approximately 55 % of each hemicellulose had been dissolved. For the succeeding stages it was accordingly found expedient to use a fresh supply of enzyme.

In each case the combined residues (4.60 g.) from four separate 2.5 g. samples which had reached the 95 hr. stage were collected, dried and subsequently digested with fresh takadiastase for an additional 45 hr., making the total time of digestion 140 hr. After determining the loss in weight and the specific rotation of the residue ( $-97.5^\circ$ ) the hydrolysis was extended in stages to 185 and finally to 210 hr., by which time approximately 94 % of each hemicellulose had been dissolved. The ultimate residues included traces of insoluble matter from the takadiastase.

The material brought into solution by the enzyme consisted in every case of (1) a soluble polysaccharide which showed  $[\alpha]_D^{20} = -51.2^\circ$  (ash-free) (in water,  $c=2$ ) and (2) a reducing sugar. Separation of the mixture was difficult and the

sugar fraction frequently contained some of the soluble polysaccharide together with traces of residual takadiastase and its specific rotation accordingly varied, values ranging from  $+10^\circ$  to  $+17.2^\circ$  being recorded. The sugar was estimated as glucose by the method of Lane & Eynon [1934] and identified as *d*-xylose in that the characteristic boat-shaped crystals of cadmium bromide-cadmium xylonate and xylose phenylosazone, M.P.  $163^\circ$  [Fischer & Paulus, 1935], were obtained. The presence of no other sugar could be detected. It is noteworthy that at all stages of the enzymic hydrolysis xylose and soluble polysaccharide were produced in the constant proportion of 3 parts by weight of the former to 2 parts of the latter.

Table I. *Action of takadiastase on hemicellulose A from oak wood*

Time of hydrolysis hr.	Loss in wt. % hemicellulose from	
	Sapwood (after removal of anhydroglucose residues)	Heartwood
48	24.77	25.0
70	37.8	36.5
76	40.0	42.6
95	55.0	54.1
140	73.8	Not determined
144	Not determined	74.9
185	86.2	85.1
193	88.0	Not determined
210	94.3	93.7

(2) *Hydrolysis by dilute mineral acids*

In order to determine whether the results obtained with takadiastase could be reproduced by means of mild hydrolysis with mineral acids the following experiments were carried out on hemicellulose A of  $[\alpha]_D^{20} = -97.5^\circ$ . The results have been calculated on an ash-free basis.

(1) A 1.2530 g. sample, on hydrolysis with 200 ml.  $N/200$   $\text{HNO}_3$  at  $100^\circ$  for 1 hr. according to the method of Bywater *et al.* [1937], lost 56 % of its weight, 36.65 % being accounted for as soluble polysaccharide of  $[\alpha]_D^{20} = -51.2^\circ$  and 12.62 % as *d*-xylose. The specific rotation of the unhydrolysed residue was  $-97.5^\circ$ .

(2) 1.4941 g. hydrolysed with 200 ml. 0.05 %  $\text{H}_2\text{SO}_4$  for 1 hr. lost 58 % of its weight, 37.5 % being accounted for as soluble polysaccharide of  $[\alpha]_D^{20} = -51.2^\circ$  and 12.29 % as *d*-xylose. Neutralization of the acid was effected in this case by  $\text{BaCO}_3$  instead of  $\text{Na}_2\text{CO}_3$ . The unhydrolysed residue gave, as before,  $[\alpha]_D^{20} = -97.5^\circ$ .

(3) Hydrolysis of a 2.0 g. sample with 200 ml. 1 %  $\text{H}_2\text{SO}_4$  for 1 hr. at  $100^\circ$  resulted in the solution of 80 % of the hemicellulose. 20 % was accounted for on neutralization as a Ba salt ( $[\alpha]_D^{20} = +49^\circ$ ) and 52 % as *d*-xylose ( $[\alpha]_D^{20} = +18.9^\circ$ ). The Ba salt was acid to phenolphthalein and gave a positive naphthoresorcinol test for uronic acids and their anhydrides. A  $\text{CO}_2$  estimation (6.99 %) on the Ba salt indicated that it was probably derived from a dixylonodimethyluronic acid [Sands & Gary, 1933]. The specific rotation of the unhydrolysed residue was  $-95.3^\circ$  in 2 %  $\text{NaOH}$ .

(4) A 1 hr. hydrolysis with 200 ml.  $N/200$   $\text{HNO}_3$  at  $100^\circ$  of 1.50 g. sapwood hemicellulose A of  $[\alpha]_D^{20} = -65^\circ$  (in 2 %  $\text{NaOH}$ ,  $c=0.4$ ) which had previously been partially fractionated with water at  $100^\circ$  [O'Dwyer, 1937] and which gave the blue colour reaction with iodine, resulted in the liberation and solution of 62 % of the starting material. The unhydrolysed residue still gave the blue

colour reaction with iodine and the soluble polysaccharide formed on hydrolysis which represented 57 % of the starting material gave a reddish purple colour with iodine, indicating that it probably contained partially hydrolysed anhydroglucose residues. It had  $[\alpha]_D^{20} = -35^\circ$  (in water,  $c=0.4$ ). No sugar was isolated in this case.

(5) 1 %  $\text{H}_2\text{SO}_4$  under the same conditions as in experiment (4) caused the liberation of 70 % of a 2.0 g. sample of hemicellulose. The residue, which gave a green colour with iodine indicating that it still contained anhydroglucose residues, had a specific rotation of  $-80^\circ$  in 2 %  $\text{NaOH}$  ( $c=0.4$ ). A Ba salt of  $[\alpha]_D^{20} = +37.5^\circ$  representing 20 % of the starting material was obtained from the hydrolysate on neutralization. The hydrolysate reduced Fehling's solution strongly and gave a positive naphthoresorcinol test. 35.7 % of the starting material was accounted for as reducing sugars estimated as glucose [Lane & Eynon, 1934]. The specific rotation of the sugar solution was  $+29^\circ$  which suggests that both glucose and xylose were present.

### (3) Analysis of soluble polysaccharide

The soluble polysaccharide obtained from hemicellulose A of  $[\alpha]_D^{20} = -97.5^\circ$  on hydrolysis with takadiastase,  $N/200$   $\text{HNO}_3$  and 0.05 %  $\text{H}_2\text{SO}_4$  respectively, was isolated as an impalpable white powder and purified by solution in water and reprecipitation with excess  $\text{EtOH}$ . The purified product gave no colour reaction with iodine and only induced faint reduction of Fehling's solution. Since the products obtained by means of all three hydrolysing agents had the same specific rotation ( $-51.2^\circ$ ) they were assumed to be identical. The analysis of a combined sample is given in Table II.

Table II. Analysis of soluble polysaccharide obtained from hemicellulose A on hydrolysis

	Uronic anhydride	Methoxyl % ash-free	Xylan	Ash %	Specific rotation (in water $c=0.5$ )
	17.90	3.08	81.75	0.3	$-51.2$
Theoretical molecule consisting of six anhydroxyloses and one methylhexuronic acid [Sands & Gary, 1933]	17.83	3.14	80.24	---	—

The yield of  $\text{CO}_2$  obtained on hydrolysis with 12 %  $\text{HCl}$  was determined by the method described by Dickson *et al.* [1930] in an improved form of apparatus evolved at this Laboratory [Campbell *et al.* 1938]. Campbell *et al.* [1938] have recently found that, when this modified apparatus is used, a number of carbohydrates, including xylose, give small but significant yields of  $\text{CO}_2$ , so that the result for uronic anhydride containing one methoxyl group (Table II) may be somewhat high.

Xylan was estimated by the Tollens procedure after correcting for the phloroglucide arising from uronic anhydride residues by the method of Lefèvre & Tollens [1907]. Modification of the method of these latter authors has been suggested by Norris & Resch [1935], but since the general question of the estimation of pentosans and related bodies in plant materials is still under investigation in several quarters, the original method of calculation has been adhered to in the present study. The methoxyl content was determined by Zeisel's method.

(4) *Hydrolysis of soluble polysaccharide*

4.8406 g. soluble polysaccharide of  $[\alpha]_D^{20} = -51.2^\circ$  were hydrolysed with 200 ml. 1 %  $\text{H}_2\text{SO}_4$  for 3 hr. at  $100^\circ$ . The precipitate obtained after hydrolysis (0.0160 g.) was filtered off and the clear filtrate neutralized with  $\text{BaCO}_3$  and treated as previously described [O'Dwyer, 1934]. It was found to contain 1.65 g. of a Ba salt upon removal of which crystalline *d*-xylose (2.58 g.) was obtained as before [O'Dwyer, 1934]. Found  $[\alpha]_D^{20} = +19.0$  (in water,  $c=4$ ), M.P.  $153^\circ$ , and xylosazone, M.P.  $163^\circ$  (Fischer & Paulus, 1935). The characteristic boat-shaped crystals of cadmium bromide-cadmium xylonate were also obtained. No sugar other than *d*-xylose could be detected. The analysis of the Ba salt after purification by dissolving in water, reprecipitating with excess of alcohol and drying as before [O'Dwyer, 1934] is given in Table III. The figures for the Ba salt are similar to those previously obtained for this substance [O'Dwyer, 1934].

Table III

	Uronic anhydride %	Ba %	Methoxyl %	Free aldehyde %	Specific rotation $+70^\circ$ (in water $c=0.4$ )	Methoxyl groups per uronic acid % 1.0 (approx.)
	43.10	17.1	7.11	6.9		
Theoretical for Ba salt of xylonomethylaldobionic acid	43.19	16.86	7.61	7.12	—	1.00

The free aldehyde groups were estimated by Cajori's method [1922], 3 hr. being required for complete oxidation by iodine in alkaline solution. The percentage of Ba was determined by Pregl's micro-method.

## DISCUSSION OF RESULTS

In conjunction with the findings of previous work the results in Table I afford interesting evidence which has not hitherto been adduced concerning the constitutional relationship which exists between hemicellulose A isolated from oak sapwood on the one hand and heartwood on the other. It had already been established [O'Dwyer, 1937] that anhydroglucose units form a definite proportion of the sapwood product and since that time the results of a number of experiments have convinced the author that such units are absent from the heartwood product. It is now observed that the complete removal of the glucose residues from sapwood hemicellulose A by hydrolysis with takadiastase at  $\text{pH } 4.5$  is accompanied by a change in specific rotation from an initial value of  $-65^\circ$  (in 2 %  $\text{NaOH}$ ) to  $-97.5^\circ$  for the unhydrolysed residue. It is noteworthy that this latter value is the same as that obtained for heartwood hemicellulose A as ordinarily isolated. Apart from this close similarity in rotatory power there is further strong evidence that sapwood hemicellulose A when freed of its inherent glucose residues is chemically identical with the heartwood product. Takadiastase under controlled conditions affects both products in precisely the same manner. The losses in weight (Table I) at corresponding stages of hydrolysis are closely comparable and the products of hydrolysis at all stages are the same.

It is observed that, chiefly on account of its greater severity, mild acid hydrolysis does not afford the same degree of insight into the structure of hemicellulose A as does the enzymic hydrolysis. Part of the products of hydrolysis is decomposed, presumably to furfuraldehyde, by the acids, and the removal of

glucose residues from the sapwood product by such reagents is accompanied by pronounced decomposition of this type.

It would now appear to be conclusive that the only constitutional change in hemicellulose A which is involved in the transformation of sapwood into heartwood consists in the removal of anhydroglucose units. It is further established that the simplest building units which are common to hemicellulose A from both sources are xylose and a monomethylhexuronic acid. It is calculated that the recurring unit of heartwood hemicellulose A probably consists of eleven of the former and one of the latter. The corresponding unit of the sapwood product is calculated to consist of one glucose, one methylhexuronic acid and eleven xylose residues.

The next step towards establishing the constitution of hemicellulose A should naturally consist in the determination of the nature of the linkages between the units which have here been identified. For instance it is of importance to determine whether the glucose units in the sapwood product are of the  $\alpha$  or  $\beta$  configuration in order to decide whether hemicellulose A is structurally related to starch on the one hand or cellulose on the other. The colour reaction of the sapwood product with iodine, coupled with the observation that glucose is obtained on hydrolysis with takadiastase, tend to indicate that  $\alpha$ -glucose residues are present, but it is freely admitted that this evidence is in itself insufficient to warrant a definite conclusion.

#### SUMMARY

1. Hemicellulose A of oak sapwood after removal of anhydroglucose residues by takadiastase has been found to be chemically identical with the product from heartwood.
2. Prolonged hydrolysis with takadiastase results in a complete fractionation of hemicellulose A into a soluble polysaccharide and xylose, 2 parts by weight of the former being formed for every 3 parts of the latter.
3. Mild hydrolysis of hemicellulose A by dilute mineral acids gives rise to the same substances as takadiastase digestion, but the yields are smaller and the proportions of sugar and soluble polysaccharide are not constant.
4. The soluble polysaccharide gives rise on dilute mineral acid hydrolysis to a monomethylhexuronate and xylose, and its determined composition agrees with that of a theoretical molecule consisting of six anhydroxylose units and one methylhexuronic anhydride unit.

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# XC. THE EFFECT OF CALCIUM ION ON TISSUE RESPIRATION; WITH A NOTE ON THE ESTIMATION OF OXALOACETIC ACID

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THAT the respiration of minced muscle is strongly inhibited by  $\text{Ca}^{++}$  in approximately "physiological" concentration was shown by Thunberg [1909, 1, 2], an observation confirmed and extended by others, including Meyerhof [1919], Holck [1934], Greville [1936] and Krebs & Eggleston [1938].  $\text{Ca}^{++}$  inhibits the respiration of other tissues also, provided that they are minced or otherwise mechanically damaged [Warburg, 1914; Holck, 1934; Krebs & Eggleston, 1938]. The contrast between the effects of neutral salts on the respiration of minced pigeon breast-muscle and cerebral cortex slices supported the view that the effect of  $\text{Ca}^{++}$  with the former tissue is due to irreversible damage consequent on its penetration into the tissue [Greville, 1936]. That in minced tissues " $\text{Ca}^{++}$  may reach intracellular enzymes to which they normally have no access" was considered also by Krebs & Eggleston [1938].

Although it has been found that the respiration in presence of fumarate was strongly inhibited by  $\text{Ca}^{++}$  [Greville, 1936], it is not certain whether this ion inhibits one or both of the central reactions of respiration, namely the formation and the removal of oxaloacetate [Annau *et al.* 1935: 1936; Laki *et al.* 1937; Krebs & Johnson, 1937]. Elliott & Elliott [1939] write: "Banga [1935] mentioned that in Ringer's solution the reduction of oxaloacetate to malate by muscle suspension was inhibited. This was probably due to the Ca in her Ringer's solution and her observation may help to fix the point of action of Ca." This remark suggested to the present writer that some hitherto unpublished experiments made by him on this matter may be of interest.

## EXPERIMENTAL

*Oxidation of fumarate.* Following Banga [1935] the suspension of minced pigeon breast muscle in phosphate was shaken at 38° for 10–15 min. aerobically in the presence of arsenite, and then for a further 15 min. after the addition of fumarate. The mixture was deproteinized with trichloroacetic acid, this and all further operations being conducted in ice-cooled vessels. After filtration a weighed aliquot was brought to pH 4·5 with NaOH, using "4·5" indicator, and the oxaloacetic acid in it was estimated by the aniline citrate method described below. In this way the oxaloacetate formation was determined in the presence

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and absence of  $0.0021 M$   $\text{Ca}^{++}$  together with  $0.0031 M$   $\text{K}^+$ . A typical experiment was arranged as follows:

Vessel no.	...	1	2	3	4
Muscle 1: 4 in $0.177 M$ phosphate, pH 7.3 (ml.)		1.5	1.5	1.5	1.5
$\text{As}_2\text{O}_3$ , $0.1 M$ (ml.)		0.5	0.5	0.5	0.5
Fumarate, $0.02 M$ (ml.)		1.0	1.0	1.0	1.0
$\text{CaCl}_2$ , $0.103 M$ (ml.)		—	—	0.08	0.08
$\text{KCl}$ , $0.155 M$ (ml.)		—	—	0.08	0.08
$\text{NaCl}$ , $0.9\%$ (ml.)		0.16	0.16	—	—
Water (ml.)		0.84	0.84	0.84	0.84
Time of incubation after fumarate addition (min.)		0	15	0	15

The results are summarized below, most of the figures giving the mean of duplicate observations. The "preformed" oxaloacetic acid (vessels 1 and 3) was never larger than  $0.026$  mg. In Exp. 5 the  $\text{Ca}^{++}$  and  $\text{K}^+$  were added with the fumarate; otherwise they were present from the start. It is seen that in the presence of  $\text{Ca}^{++}$  and  $\text{K}^+$  the oxaloacetate formation is strongly inhibited.

Exp. no.	Oxaloacetic acid formed (mg.)	
	Ca, K absent	Ca, K present
1	0.43	0.06
2	0.36	0
3	0.11	0.03
4	0.24	0.05
5	0.29	0.13

When the muscle was suspended in the  $\text{NaHCO}_3$ -containing salt solution used by Annau *et al.* [1935], but without the Ca, there was a good oxaloacetic acid formation from fumarate in the presence of arsenite.  $\text{Ca}^{++}$  addition ( $0.003 M$ ) caused a strong inhibition, whether the flasks were filled with air, or with air containing  $5\%$   $\text{CO}_2$ .

*Oxaloacetate removal.* Banga's finding, referred to above, was obtained with the "semi-quantitative" Simon-Piaux nitroprusside test. In the experiments summarized below, minced pigeon breast muscle was shaken for 15 min. aerobically at  $38^\circ$  in the presence and absence of  $\text{Ca}^{++}$  and  $\text{K}^+$  (concentrations as above), and then for a further 15 min. after the addition of 15 mg. neutralized oxaloacetic acid. Oxaloacetic acid was determined by the aniline citrate method before and after the second incubation. It will be seen that the oxaloacetate removal is inhibited by the added cations. The total amount of carbonyl compound present was also determined [Clift & Cook, 1932]; and it was found that at the end of the experiment the amount present was greater than the residual amount of oxaloacetic acid. The difference was presumed to be due to the presence of pyruvic acid [Banga & Szent-Györgyi, 1937], especially as the com-

Exp.	Wt. of minced muscle (g.)	Added cations	Oxaloacetic acid dis- appearance (mg.) (1)	"Pyruvic acid" formed (mg.) (2)	Minimum oxaloacetic acid removal by ways other than decarboxylation, from (1) and (2) (mg.) (3)
1	0.38	—	8.8	3.0	4.4
		$\text{Ca}^{++}$ , $\text{K}^+$	4.8	2.3	1.3
2	0.25	—	3.3	1.3	1.3
		$\text{Ca}^{++}$ , $\text{K}^+$	2.1	1.1	0.5
3	0.38	—	8.8	2.0	5.8
		$\text{Ca}^{++}$ , $\text{K}^+$	6.3	1.9	3.4



pound formed was alkali-stable [Clift & Cook, 1932]. The pyruvate formation, which was probably due to decarboxylation of oxaloacetate, was not greatly affected by the  $\text{Ca}^{++}$  and  $\text{K}^+$ .

*Succinate formation.* Straub found that in Ringer solution the aerobic disappearance of fumarate in the presence of malonate was much less than in phosphate; under these conditions and in the latter medium Gözsy found succinate formation [Annau *et al.* 1935]. In the two experiments summarized below, an inhibition of succinate formation by 0.0018 *M*  $\text{Ca}^{++}$  with 0.0028 *M*  $\text{K}^+$  was observed.

Time 30 min. Air. Minced muscle 1 g. 38°. Malonate 0.01 *M*. Fumaric acid 7 mg. (neutralized).

Succinic acid formed (mg.)

Exp.	$\text{Ca}^{++}$ , $\text{K}^+$ absent	$\text{Ca}^{++}$ , $\text{K}^+$ present
1	1.66	0.75
2	1.88	0.92, 0.81

The succinic acid was estimated as follows: after deproteinization with alcohol, the acidified solution was extracted with ether in a continuous extractor, the dry extract was autoclaved to remove malonate, and the succinic acid determined using a succinoxidase-containing dispersion obtained from pigeon breast muscle, which did not oxidize lactate,  $\alpha$ -ketoglutarate or glycerophosphate [cf. Annau *et al.* 1935; Weil-Malherbe, 1937].

#### *Estimation of oxaloacetic acid*

In Ostern's [1933] method the  $\text{CO}_2$  evolved when aniline reacts with oxaloacetic acid is determined in the Warburg apparatus. The analysis is performed at 5° in order to minimize the breakdown of the acid before the addition of the aniline. However, at this temperature the reaction is slow, becoming complete in 60–90 min. Two ways have been used to increase the amount of dissolved aniline and hence the speed of the reaction.

(1) *Citrate method.* Edson [1935] used aniline citrate in the manometric determination of acetoacetic acid at 25°. His technique can be applied to the estimation of oxaloacetic acid at 5°. The vessel is shaken for 10 min. before the addition of the aniline. If the solution originally contained much bicarbonate, the shaking should be continued for a test period of 5 min. Reaction is complete in 10–20 min. after addition of aniline citrate from the side-bulb. For the calculation of vessel constants it is necessary to know the solubility of  $\text{CO}_2$  in the mixture in the vessel. For this  $\alpha_{\text{CO}_2}^{\text{ox}}$  was found to be 1.15. If it is necessary to use rather more oxaloacetic acid solution in the vessel, the value  $\alpha_{\text{CO}_2}^{\text{ox}} = 1.19$  should be used for the mixture 3 ml.  $\text{H}_2\text{O}$  + 0.4 ml. 50 % citric acid + 0.4 ml. aniline citrate solution. Duplicate determinations never differed by more than 2.5 %. 97 % purity was indicated by the method for three different oxaloacetic acid preparations.

(2) *Alcohol method.* The bulb contains 0.2 ml. aniline previously mixed with 0.14 ml. conc.  $\text{HCl}$ . The main part contains 1.5 ml. absolute alcohol, 0.3 ml. acetate buffer (0.3 *N* Na acetate + 2.7 *N* acetic acid) and 0.86 ml. solution to be analysed. The thermo-barometer contains 0.86 ml. of water instead of solution. The analysis is carried out as in method (1). The reaction is complete in 10–15 min. at 5°.  $\alpha_{\text{CO}_2}^{\text{ox}} = 1.21$ . The method gives the same results on oxaloacetic acid solutions as do Ostern's method and method (1).

*Acetoacetic acid.* The alcohol method does not distinguish between oxaloacetic and acetoacetic acids. The reaction with acetoacetic acid at 5° is complete in

10–15 min. (with the citrate method the reaction at  $25^\circ$  takes up to 70 min. [Edson, 1935]). With the citrate method at  $5^\circ$ , however, oxaloacetic acid can be determined in the presence of acetoacetic acid.<sup>1</sup> The  $\text{CO}_2$  evolution with the latter is slow and fairly constant for a long time, and extrapolation to zero time will give the amount of oxaloacetic acid with reasonable accuracy (Fig. 1). Nevertheless it is possible that the alcohol method may also prove convenient on occasion.

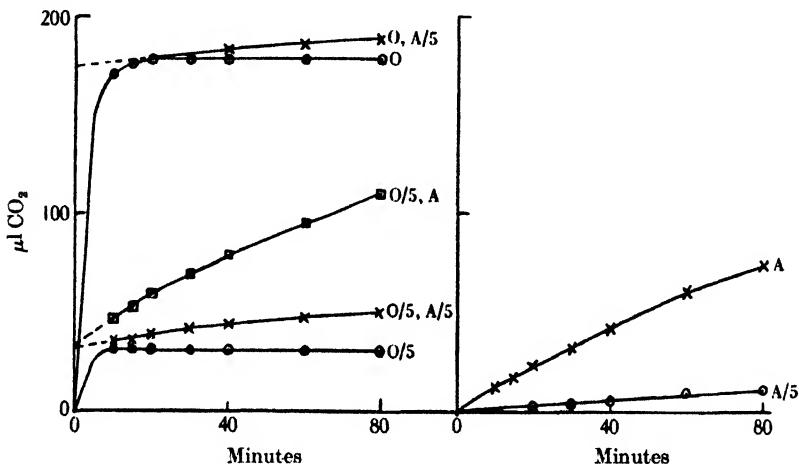


Fig. 1. A = 1 ml., A/5 = 0.2 ml., acetoacetic acid. O = 1 ml., O/5 = 0.2 ml., oxaloacetic acid. O/5, A/5 = 0.2 ml. oxaloacetic + 0.2 ml. acetoacetic acid. O/5, A = 0.2 ml. oxaloacetic + 1 ml. acetoacetic acid. O, A/5 = 1 ml. oxaloacetic + 0.2 ml. acetoacetic acid. (Oxaloacetic and acetoacetic acid solutions approx. 1 mg. per ml.)

**Thermostat.** A simple and inexpensive device serves to keep the thermostat at  $5^\circ$ . Water is transferred to the thermostat from a bucket containing ice by means of a small water-circulating pump. It returns through a syphon tube. The pump is driven by an electric motor which is switched on and off by a relay controlled by a mercury-toluene regulator in the thermostat. The occasional addition of a lump of ice to the bucket is the only attention necessary.

### SUMMARY

1. On the addition of  $\text{Ca}^{++}$  and  $\text{K}^{+}$  in physiological salt solution concentrations to a suspension of minced muscle, both the formation and the removal of oxaloacetic acid, and also the accumulation of succinic acid, are inhibited. Hence the inhibitory effect of  $\text{Ca}^{++}$  on the respiration of minced muscle cannot be localized in any particular enzymic reaction.

2. Rapid methods are given for the manometric estimation of oxaloacetic acid.

The writer wishes to thank Prof. E. C. Dodds for the interest he has taken in this work.

<sup>1</sup> Elliott & Elliott [1939] have used Edson's method at  $38^\circ$  in order to determine oxaloacetic acid. At this temperature the method does not differentiate between oxaloacetic and acetoacetic acids.

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# XCI. A COMPARISON OF THE METABOLIC PATHWAYS OF GLYCINE AND ALANINE

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RINGER & LUSK [1910] gave glycine to a fed phlorhizinized dog and observed the elimination in the urine of extra glucose which they considered was derived from the conversion of glycine into glucose. On the other hand, by giving glycine or alanine intravenously to cats under chloralose, Reid [1936] showed that liver glycogen readily increased with alanine but not with glycine. Bach & Holmes [1937], using the liver slice technique, found that glycine did not increase gluconeogenesis in the liver. Stohr [1938] obtained a decrease and sometimes an increase in liver glycogen when glycine was given to fasting rats. Results of other experiments by the author are now available. These appear to support the previous conclusions of the author [1936] and of Bach & Holmes [1937] that the metabolism of glycine does not follow the carbohydrate pathway.

## EXPERIMENTAL

Non-anaesthetized animals, rats and dogs, were used in the following experiments.

### (1) EXPERIMENTS ON RATS

Standard male white rats were used of about 150–250 g. weight. They were deprived of food but not of water for 24 hr. before use. They were then fed by

Table I. *Effect of giving 2.23 ml. of solutions of alanine and of glycine on the store of glycogen in the livers of fasted rats*

Exp.	No. of rats	Liver glycogen as glucose		Blood sugar (mg. 100 ml.)
		Total (mg.)	%	
		(a) By stomach tube		
Control	11	3.9 (1.5–7.5)	0.08 (0.05–0.15)	78 (72–88)
Alanine 20%	15	48.2 (32–95)	0.84 (0.55–1.33)	103 (98–107)
Glycine 16.48%	14	2.7 (2.0–3.1)	0.06 (0.04–0.08)	104 (101–108)
Glycine 24.72%	4	3.0 (2.0–3.5)	0.06 (0.05–0.08)	—
		(b) By intraperitoneal injection		
Control	4	3.6 (2.5–4.5)	0.06 (0.05–0.07)	—
Alanine 20%	8	51.4 (37–80)	0.83 (0.49–1.28)	—
Glycine 24.72%	8	4.3 (3.2–5.5)	0.07 (0.06–0.08)	—
		( 723 )		

stomach tube with 2.23 ml. (volume of fluid delivered by the 2 ml. syringe with the piston pulled back to the stop) of one of the following solutions: (1) alanine 20%; (2) an equimolecular solution of glycine, viz. 16.48%; (3) a solution of glycine, viz. 24.72%, containing the same number of carbon atoms as the 20% solution of alanine. In some experiments the amino-acids were given intraperitoneally. Larger volumes of these hypertonic solutions given in this way usually proved fatal.

Four hr. after feeding, the rats were killed rapidly in a coal gas chamber, samples of mixed blood obtained from the neck blood vessels, and sugar percentages determined by the method of Hagedorn & Jensen [1923]. The whole liver was removed and its glycogen content determined as glucose by the method of Good *et al.* [1933]. Controls were given the same volume of 3% NaCl. The results are shown in Table I.

## (2) EXPERIMENTS ON DOGS

The experiments on dogs allowed the metabolic pathways of glycine and alanine to be viewed from a different standpoint from that of the formation of glycogen in the liver. When carbohydrate is given to a normal fasting animal, the excretion of N and of inorganic S decreases, showing that the breakdown of protein is spared. On the other hand, when an amino-acid is fed, the increased metabolism associated with its disposal, i.e. its specific dynamic action, requires increased production of energy, which, in the case of the fasting animal, is furnished by increased usage of fat and protein. If the deaminated residue forms sugar, the breakdown of protein should be less than in the case of a non-sugar-forming amino-acid provided that their specific dynamic actions are of the same order. From the experiments on rats it appears that alanine provides a sugar-forming deaminated residue but not glycine. Their specific dynamic actions per molecule are of the same order [Rapport & Beard, 1927]. Consequently, when the amino-acids are fed in equimolecular amounts to fasting animals, one would expect the breakdown of tissue protein to be less for alanine than for glycine. Results of observations on the N excretion in the urine after amino-acid feeding would be difficult to assess for obvious reasons, but the behaviour of the excretion of inorganic S might give information concerning the metabolic pathways since both amino-acids are sulphur-free.

### *Methods*

Two bitches, weights 10–11 and 14–15 kg., were prepared for easy catheterization and used throughout the experiments. The following routine was adopted for each experimental period. After a preliminary period of deprivation of food, but not of water, lasting 48 hr. followed by emptying of the bladder, the animals were fed with glucose, alanine, glycine or water and placed in a metabolism cage for 50 hr. At the end of this period the bladder was emptied and the total N and inorganic S excreted during the 50 hr. period determined.

The dogs were used in the above way not oftener than once every 2 weeks. Weight charts were kept to ensure that their body weight remained normal despite repetition of the fast periods. Observations made during oestrus and also in the 2 or 3 weeks before and after oestrus have been excluded from the results because the metabolism, as judged by the excretion of N and inorganic S, appears to be increased before and during oestrus.

The results for one of the dogs are summarized in Table II.

Table II. *Effect on the amount of inorganic S excreted during 50 hr. after feeding glucose, alanine, glycine or water to a bitch, weight 10–11 kg., previously fasted for 48 hr.*

Substance fed	No. of exp.	Total N g.	Urine	Inorganic S g.
			(Total N) – (N fed) g.	
Water	8	4.62 (4.3– 4.7)	4.62	0.159 (0.153–0.163)
Glucose 50 g.	8	4.02 (3.88– 4.17)	4.02	0.140 (0.134–0.145)
Glycine 25 g.	8	9.87 (9.61–10.20)	5.21 (4.95–5.54)	0.230 (0.214–0.246)
Alanine 30 g.	8	8.57 (8.29– 8.78)	3.95 (3.67–4.16)	0.190 (0.181–0.204)

### DISCUSSION

The experiments on rats show that alanine readily increases the store of glycogen in the liver whereas glycine does not do so. This finding holds good whether the amino-acids are absorbed through the intestinal tract or the peritoneum. Since the specific dynamic action per molecule of glycine and of alanine is of the same order, a greater intensity of the specific dynamic action cannot account for the failure of glycogen deposition in the case of glycine.

Under the standard conditions maintained in the experiments on dogs it has been shown that glucose decreases the excretion of inorganic S during the period of fasting dealt with. Both alanine and glycine increase the excretion of inorganic S presumably because the specific dynamic action associated with the disposal of these amino-acids increases the catabolism of protein. The increase, however, is less for alanine probably because alanine forms sugar and so saves partially the increased catabolism of protein which would otherwise occur during the specific dynamic action of the amino-acid.

### SUMMARY

The metabolic pathways of the deaminated residues of glycine and of alanine have been compared indirectly by two different methods.

Absorbed from the intestine or peritoneum, alanine readily increases the store of glycogen in the livers of fasting rats, whereas glycine does not do so.

When these amino-acids are fed to fasting dogs, alanine causes a smaller rise in the catabolism of protein than glycine presumably because alanine but not glycine forms sugar and so saves protein.

It is suggested, therefore, that the metabolic pathways of the deaminated residues of alanine and of glycine are different.

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# XCII. CO<sub>2</sub> UTILIZATION DURING THE DISSIMILATION OF GLYCEROL BY THE PROPIONIC ACID BACTERIA<sup>1</sup>

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WOOD & WERKMAN [1936] reported that propionic acid bacteria reduced CO<sub>2</sub> (obtained from CaCO<sub>3</sub>) during the dissimilation of glycerol. They found that the amount of CO<sub>2</sub> present following fermentation was much less than the amount that had been added as CaCO<sub>3</sub>. They also found that the total organic C content of the fermented culture was greater than the total organic C content of the unfermented medium [Wood & Werkman, 1938]. These were the first reports concerning such an unusual metabolic reaction of the propionic acid bacteria. Barker [1936] and van Niel [1937] seemed reluctant to accept, without confirmation, the original report regarding the propionic acid bacteria. However, the later evidence presented by Wood & Werkman [1938] should remove all doubts.

During the course of other work upon the propionic acid bacteria an indication of CO<sub>2</sub> utilization was noted and the more carefully controlled experiments recorded here were performed. In view of the unusual nature of this reaction, the controversial interest accorded the first reports and the desirability, as suggested by Wood & Werkman, of confirmation by other laboratories, it seemed worth while to report these results.

## EXPERIMENTAL

### *Cultures, medium and methods*

The cultures were *Propionibacterium pentosaceum*, P<sub>11</sub> (van Niel's 4 and Wood & Werkman's 49 W); *P. technicum*, P<sub>12</sub> (van Niel's 22); and *P. shermanii*, P<sub>19</sub> (Wood & Werkman's 52 W).

The medium contained 20 g. glycerol, 20 g. CaCO<sub>3</sub>, 500 ml. yeast water,<sup>2</sup> and distilled water to make 1 l. Equivalent amounts of sterile CaCO<sub>3</sub> were added to the flasks just before inoculation. A 3 % inoculum of washed 48 hr. cells from a glucose-yeast water medium was used. Air in the system was displaced with O<sub>2</sub>-free N<sub>2</sub> immediately after inoculation. These fermentations were incubated at 30° for 28 days.

The residual glycerol, following extraction from the culture medium, was determined by the method of Woolley (unpublished) which is a modification of the method of Wagenaar [1911]. CO<sub>2</sub> was determined on both the culture and a sterile control. The CO<sub>2</sub> produced during fermentation and that liberated by

<sup>1</sup> Supported in part by a grant from the Wisconsin Alumni Research Foundation. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

<sup>2</sup> The clear extract from 200 g. pressed yeast autoclaved in 1 l. water.

acidification with H<sub>3</sub>PO<sub>4</sub> and removed by aeration at the end of the fermentation was absorbed in KOH solution. The residual KOH was titrated after the addition of an excess of BaCl<sub>2</sub>. The CO<sub>2</sub> utilized during the fermentation was then equivalent to the CO<sub>2</sub> from the CaCO<sub>3</sub>, as determined from the control flask, minus the CO<sub>2</sub> from the fermentation flask. Volatile acids, propionic and acetic, were determined by a modified Duclaux distillation after steam distillation from an acidified (pH 2) aliquot of the culture. The non-volatile acids, lactic and succinic, were removed from the residue of the volatile acid distillation by continuous extraction with ethyl ether. Lactic acid was determined by the method of Friedemann & Graesser [1933] and succinic acid by the following modification of the method of Moyle [1924]. An acidified aliquot of the residue of the volatile acid distillation, containing 10–75 mg. succinic acid, was extracted with ethyl ether for 24 hr. The extract was taken up in 30–40 ml. water containing 1 ml. 95 % H<sub>2</sub>SO<sub>4</sub>, heated and 0.1N KMnO<sub>4</sub> added until a permanent brown precipitate appeared in the boiling solution. This oxidation destroyed the lactic acid. The solution was evaporated to a convenient volume, cooled and extracted with ethyl ether for 24 hr. The extract was taken up in 15–20 ml. water and 5 ml. 10 % AgNO<sub>3</sub> added. This solution was adjusted to pH 6.0–6.5 with 0.5N NH<sub>4</sub>OH and bromocresol purple (other indicators in this range are unsatisfactory), filtered on a Gooch crucible, and washed with four or five 3 ml. portions of 50 % alcohol. The precipitate was dissolved with two 10 ml. portions of hot 1:4 HNO<sub>3</sub>, cooled, and titrated with 0.07N KCNS and saturated ferric alum indicator. Two mol. of KCNS are equivalent to one mol. of succinic acid. Recovery of known samples averaged 98–99 %.

## RESULTS

The data are recorded in Table I. Uniformly the principal course of the reaction has been the production of propionic and succinic acids accompanied by small amounts of acetic and lactic acids. In each fermentation there has been

Table I. *Glycerol dissimilation by the propionic acid bacteria*

Culture No.	P <sub>11</sub>	P <sub>11</sub>	P <sub>12</sub>	P <sub>12</sub>	P <sub>13</sub>
Glycerol fermented mM. per l.	205.3	212.8	183.9	186.5	92.5
CO <sub>2</sub> utilized per 100 mM. fermented glycerol	21.40	25.6	9.22	14.36	25.41
Products per 100 mM. fermented glycerol					
Propionic acid	75.50	70.00	87.14	90.07	66.26
Acetic acid	2.92	8.75	0.92	0.99	5.84
Succinic acid	23.60	23.78	9.90	13.21	25.48
Lactic acid	—	—	0.27	0.99	0.70
C recovery (%)					
Basis, glycerol plus CO <sub>2</sub>	101.8	99.2	98.2	104.2	96.7
Basis, glycerol	108.9	107.5	101.2	109.3	105.0
Oxidation-reduction index					
Basis, glycerol plus CO <sub>2</sub>	1.04	1.02	1.04	0.95	1.05
Basis, glycerol	1.64	1.77	1.26	1.26	1.89

a definite utilization of CO<sub>2</sub>, a fact which may be demonstrated in several ways. The strongest evidence appears in the CO<sub>2</sub> determinations. In every case the total CO<sub>2</sub> remaining in the flask and absorber following fermentation is less than the amount of CO<sub>2</sub> originally added as CaCO<sub>3</sub>. In the fermentation showing the smallest CO<sub>2</sub> utilization the observed difference in CO<sub>2</sub> concentrations is some 25 times the experimental error; in other fermentations the observed CO<sub>2</sub> uptake is well over 100 times the experimental error.



Supplementary evidence is offered by the C recoveries and the oxidation-reduction indices. When the C recoveries are based upon glycerol alone they are uniformly high, for the C content of the products is greater than the C content of the glycerol fermented. However, when the C recoveries are based upon glycerol plus CO<sub>2</sub> the values approach 100 %. The oxidation-reduction indices indicate a large excess of oxidized products when calculated upon the basis of glycerol alone. However, if the utilized CO<sub>2</sub>, an oxidized compound, is entered in these calculations, the oxidation-reduction indices approach the ideal value of 1.00.

In each of these fermentations, as in most of those reported by Wood & Werkman, the succinic acid is, on a molar basis, approximately equal to the CO<sub>2</sub> utilized.

#### SUMMARY

The report of Wood & Werkman that the propionic acid bacteria utilize CO<sub>2</sub> during the dissimilation of glycerol has been confirmed.

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# XCIII. ESTIMATIONS OF IRON IN THE LYMPH GLANDS OF MICE DURING TREATMENT WITH A CARCINOGENIC COMPOUND

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PARSONS and her fellow-workers [Mayneord & Parsons, 1937; Clarkson *et al.* 1938; Parsons, 1938] have reported the deposition of Fe in the lymph glands of mice undergoing treatment with the water-soluble carcinogenic substance sodium 1:2:5:6-dibenzanthracene-9:10-*endo*- $\alpha\beta$ -succinate [Cook, 1931]. Similar results were obtained in animals treated with other carcinogenic substances. The process involved may consist of a slow destruction of red blood cells, followed by storage of the liberated Fe in the lymph glands of the animal. Occasionally the lymph glands have the macroscopic appearance of haemolymph glands, the mesenteric lymph glands being particularly liable to this change. The water-soluble compounds used by Parsons and her co-workers have been shown to have a haemolytic action *in vitro* [Warren, 1939].

The experiments described in the present paper were carried out with a view to placing on a quantitative basis the histological demonstration of the accumulation of Fe in the lymph glands during the latent period before tumour formation. A few measurements of Fe in the lymph glands of mice bearing spontaneous tumours are recorded, and, in view of the interest of the results, this investigation is being extended.

## METHODS

The determination of Fe in biological material is notoriously difficult. The most serious error which is likely to occur is loss of Fe during ashing on account of the volatility of ferric chloride. It became clear at an early stage of the present experiments that the variation in the Fe content of the glands of mice which had received the same dose of carcinogenic compound was great enough to make it desirable to employ as many animals as possible to obtain a reliable mean value. The method employed had to be a reasonably rapid one, even if some degree of accuracy had to be sacrificed to achieve this. With this in mind, the method described below was adopted. Control experiments with known quantities of Fe were satisfactory. With the reservation that great accuracy is not claimed for the absolute values, there is no doubt that the results give a satisfactory picture of the relative Fe contents of the lymph glands.

Stock male mice were injected thrice weekly subcutaneously in the right flank with 0.3 ml. of a 0.4% solution of the sodium salt of 1:2:5:6-dibenzanthracene-9:10-*endo*- $\alpha\beta$ -succinic acid (hereafter abbreviated as "D.B.A.-mal"). 10 mice were killed after receiving 20 doses. Further groups of 10 mice were killed at various dose intervals. One axillary gland from each side, one mesenteric and one inguinal gland (left) were dissected entire with the surrounding fat. The glands were washed free from extraneous blood with saline and dried

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roughly with filter paper. The four glands from one mouse were ashed together. The glands were transferred to ashing tubes (made by blowing bulbs 25–30 mm. diameter on 16 × 150 mm. pyrex test tubes). 0.5 ml. of a mixture of conc.  $\text{HNO}_3$  (3 parts) and conc.  $\text{H}_2\text{SO}_4$  (1 part) was added to the glands and carefully evaporated. This process was repeated until no carbonaceous material remained. In practically all cases a total of 3 ml. of acid mixture was used. The residue after the final evaporation of the acid was dissolved in 2–3 ml. of water and transferred to a 10 ml. standard flask. The Fe was estimated colorimetrically by measurement of the colour produced by salicylsulphonic acid in the presence of ammonia. To the contents of the standard flask were added in order 2 ml. 2N  $\text{NH}_4\text{Cl}$ , 2 ml. 20% salicylsulphonic acid and 2 ml. 10% ammonia, and water to 10 ml. The purple colour at first developed in the acid solution changes to yellow or orange on the addition of the ammonia. Blank solutions were prepared by carrying out the evaporation of the appropriate amount of acid mixture without lymph gland and making up the solution with reagents and water to 10 ml.

The colorimetric measurements were made on a Hilger "Spekker" Absorptiometer. The mode of operation of this instrument has been previously described [e.g. Callow *et al.* 1938]. The instrument was calibrated by means of standard ferric alum solution employing Ilford "Spectrum Violet" filters. From Fig. 1

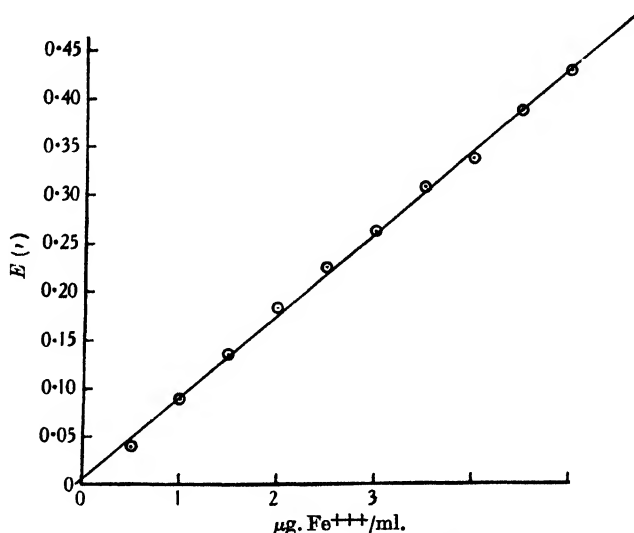


Fig. 1. Iron estimations. Calibration curve  $E(v) = 0.0077 + 0.08496 \times \mu\text{g. Fe}^{+++}/\text{ml.}$

it is seen that there is a straight line relationship between the measured absorption of violet light and the quantity of Fe present in the solution over the range of concentration employed.

## RESULTS

### (1) Normal mice

Parsons has noted that it is rarely possible to demonstrate the presence of Fe in the lymph glands of normal untreated mice by the use of the prussian blue reaction. In order to have a basis for comparison with the glands of the mice undergoing treatment with the carcinogenic substance, the total Fe in the four glands (see above) of 21 normal male mice, 6 months of age (an age comparable

with that of the experimental mice in the later stages of the injections), was determined. The amounts found were all small. The maximum value in any mouse was 6.0  $\mu\text{g}$ . and the average value approximately 3.0  $\mu\text{g}$ . The individual values are shown in Table 1. The figures denote  $\mu\text{g}$ . Fe.

Table 1. *Fe in the lymph glands of 21 untreated male mice*

2.0	2.5	2.0	1.5	5.0	4.0	3.0	1.0	3.0	2.5	1.0
4.0	4.5	6.0	3.5	4.0	3.5	4.0	2.0	2.5	4.0	

(Mean value 3.1)

(2) *Mice treated with the carcinogenic compound*

The results for 70 mice at various dose levels are given in Table II. In the case of those figures marked with an asterisk the animal showed signs of haemolymph gland formation and these values are not included in the averages. In Fig. 2 the average values are plotted against the number of doses injected before

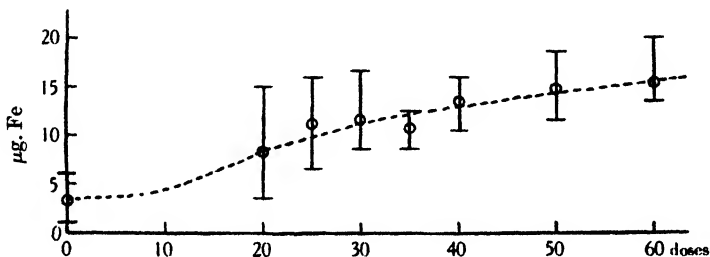


Fig. 2. Total Fe in glands of mice treated with Na-1:2:5:6-dibenzanthracene-9,10-endo- $\alpha\beta$ -succinate.

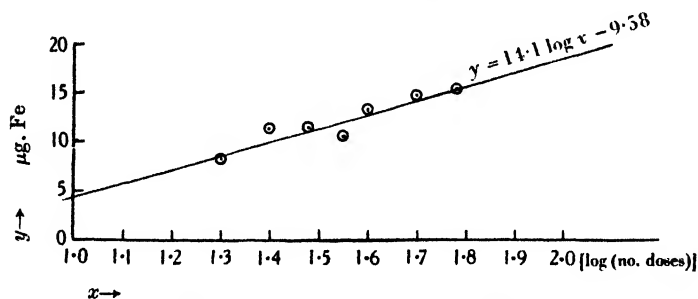


Fig. 3. Fe in glands/log (no. of doses).

Table II. *Fe in the lymph glands of 70 treated male mice*

No. of doses ...	20	25	30	35	40	50	60
Total Fe in glands of mouse, $\mu\text{g}$ .	7.00	9.0	8.5	9.0	14.0	18.0	13.5
	7.75	11.5	10.0	10.0	15.0	29.5*	13.5
	7.50	10.5	10.0	13.5	20.0*	12.5	17.0
	3.50	16.0	10.5	10.5	13.5	18.5	15.5
	15.00	13.5	9.5	10.0	16.0	13.5	14.5
	7.75	10.0	12.5	8.5	13.0	16.0	14.5
	8.25	6.5	11.0	11.5	11.5	11.5	20.0
	9.00	11.0	11.0	12.0	12.0	12.5	24.5*
	8.25	10.0	16.5	10.5	16.0	15.0	14.5
	7.50	14.0	15.5	12.5	10.5	15.0	15.0
Mean value	8.15	11.2	11.5	10.8	13.5	14.7	15.3

\* Haemolymph gland.

the animal was killed. The vertical lines indicate the range of the individual Fe values at that dose level.

In Fig. 3 the mean values from Table II are plotted against the logarithms of the number of doses injected. The seven points fall approximately on the straight line given by the equation:

$$\mu\text{g. Fe}^{+++} = 14.1 (\log \text{ no. of doses}) - 9.58.$$

The broken curve shown in Fig. 2 has been calculated from this equation. The inflexion of the curve shown between zero and 20 doses has, of course, not been experimentally determined.

During the later stages of the injections 9 mice developed sarcomas at the site of injection. Estimations of Fe in the four lymph glands of these animals gave the following values (not included in Table II): 24.0, 13.5, 15.0, 6.5, 17.5, 36.0, 10.5, 17.5, 16.5 (mean 17.4  $\mu\text{g.}$ ).

In order to be certain that the increase in total Fe in the glands of the injected mice represented a true increase per unit weight of the gland tissue it was necessary to investigate the possibility of any considerable increase in size of the glands from the injected animals as compared with the glands from normal uninjected animals. With this in view, the glands (2 axillary and 1 inguinal) from 20 normal mice and 10 mice injected with 50 doses of D.B.A.-mal. were removed, together with their fat capsules, and immersed in Bouin's fixative (saturated picric acid 75 ml., formalin 15 ml., glacial acetic acid 10 ml., urea 1 g.) for 24 hr. The three glands from each mouse were then shelled out, roughly dried and weighed. The weights in mg. of the glands from the control mice were: 17.0, 23.0, 21.5, 50.0, 21.0, 13.0, 12.0, 17.0, 21.0, 34.0, 35.0, 18.0, 12.0, 20.5, 29.0, 26.5, 45.0, 33.0, 18.0, 21.0 (mean 24.4 mg.). In the case of the injected mice the corresponding weights were: 28.0, 24.0, 28.5, 25.0, 26.5, 30.0, 24.0, 20.0, 25.5, 32.0 (mean 26.35 mg.). The difference between the two means is approximately 2.0 mg. and is not significant.

In the experiments described above the mice were killed 24 hr. after the last injection. It seemed of interest to determine whether the increased Fe deposit in the glands would be removed on discontinuing the injections. For this purpose two groups of mice which had received 48 and 54 doses respectively of carcinogenic compound were kept untreated for 3 weeks before killing. The Fe values found were: 8.0, 8.5, 6.5, 3.0, 11.0, 9.5, 8.5, 9.0 (mean 8.0  $\mu\text{g.}$ ) for the first group and 12.5, 15.0, 13.5, 11.0, 14.0, 12.5, 7.5, 12.0 (mean 12.25  $\mu\text{g.}$ ) for the second. Comparison of these values with Fig. 2 indicates that there is some removal of the accumulated Fe from the glands on discontinuing the injections. In connexion with this question of the lability of the Fe deposits it is perhaps worth mentioning that the glands from the injected mice, which were treated with Bouin fixative in the experiment described above, gave values for the Fe content indistinguishable from those for normal glands. That is, Bouin's fluid dissolves the abnormal deposit of Fe.

### (3) *Mice bearing spontaneous tumours*

An opportunity presented itself of estimating the total Fe in the lymph glands of 13 mice bearing spontaneous tumours. The animals were mainly female mice of a high cancer strain (Little Dilute Brown) bearing spontaneous mammary carcinomas. The values for total Fe in the same four lymph glands were as follows: 14.5, 18.0, 8.5, 8.5, 11.0, 13.5, 14.0, 25.0, 8.5, 14.0, 9.0, 19.0, 11.5 (mean 13.5  $\mu\text{g.}$ ). Thus the average amount of total Fe present in the lymph

glands of these animals is of the same order as the amount present in the glands of stock mice after treatment with 40 doses of the carcinogenic D.B.A.-mal. compound.

#### DISCUSSION

The results obtained with the lymph glands of mice injected with D.B.A.-mal. are in excellent agreement with the histological findings of Parsons. Seven weeks after the first injection the total Fe in the glands has risen to 3 times the normal amount: at 12 weeks the Fe content is 4 times, and at 20 weeks 5 times the normal value. The Fe content increases most rapidly during the early stages of the injections. The rise during the interval between the 20th and 40th doses is approximately twice as great as the rise between the 40th and 60th doses. Fig. 3 indicates that the relation of dose to Fe deposition is that of a normal drug action.

Evidence for the lability of the additional Fe is afforded by the fact that the glands of mice killed 3 weeks after the last injection show lower Fe contents than the glands of mice at the same dose level killed 24 hr. after the last injection.

No proof can yet be offered that the phenomenon investigated in the present work has any causal relationship with the malignant growths which arise on continued injection of the D.B.A.-mal. compound. It must be borne in mind that the haemolytic and carcinogenic actions of this substance may be two entirely distinct properties. The question is, however, of some importance and the preliminary results on mice bearing spontaneous tumours reported in the present paper appear sufficiently encouraging to warrant an extended application of the method to cancer-susceptible strains before the appearance of malignant growth.

#### SUMMARY

Estimations of the Fe contents of the lymph glands of mice undergoing injection with a carcinogenic substance have been carried out.

Increase in the total Fe content up to 5-fold the normal value has been demonstrated.

Evidence of the lability of the additional Fe has been obtained.

Preliminary experiments suggest that a similar process may occur in mice bearing spontaneous tumours.

I am indebted to Dr L. D. Parsons for much help with the injections of the animals and dissections of the lymph glands and for her constant encouragement throughout the whole course of the work. It is a pleasure to express my thanks to the Sir Halley Stewart Trust for a Fellowship held during this work and to the British Empire Cancer Campaign for generous grants which have supported this investigation.

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## XCIV. FATTY ACID OXIDATION IN LIVER

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FATTY acid oxidation in tissues has been studied by measuring the oxygen uptake or the reaction products (acetoacetic and  $\beta$ -hydroxybutyric acids). The quantitative importance of  $\beta$ -oxidation cannot be ascertained in this way, as a fraction of the acids might undergo some other type of oxidation, such as the  $\omega$ -oxidation of Verkade & van der Lee [1934].

In Knoop's classical theory the successive elimination of a two-carbon substance is required. This substance has been supposed to be acetic acid, but has never been identified.

The tissue slice technique of Warburg has been applied to the study of this problem by Edson [1935, 1, 2; 1936], Jowett & Quastel [1935, 1, 2, 3], Edson & Leloir [1936], Mazza [1936], Cohen & Stark [1938], etc. Combining this method with microestimations of fatty acids we have endeavoured to obtain more quantitative results for the oxidation of normal fatty acids with 1-8 C atoms.

Whereas liver slices rapidly oxidize fatty acids, ground tissue or enzyme preparations have never shown any such activity. For this reason it has been supposed that fatty acid oxidation is in some way dependent on cell structure. We have found it possible to prepare a cell-free liver "brei" which will oxidize butyric acid, but attempts to isolate the enzyme system have so far failed, presumably owing to a rapid inactivation by reduction of some of the components.

### METHODS

Flasks as described by Krebs [1933] were filled with 30 ml.  $\text{NaHCO}_3$ -Ringer solution [Krebs, 1933] and with rat liver slices (about 200 mg. dry wt.). After mixing the contents thoroughly, a 12 ml. initial sample was withdrawn; the flasks were then gassed with  $\text{O}_2 + 5\% \text{CO}_2$  and shaken 2 hr. at  $37.5^\circ$ .

Bicarbonate was estimated as previously described [Leloir & Muñoz, 1937].

*Ketone bodies.* Acetoacetic acid was estimated by both the manometric and modified Van Slyke methods as described by Edson [1935, 1]. The amount of NaOH given by Edson for the Rupp titration is slightly too small and may give rise to errors. It is better to double the amount, and then use a double quantity of acetic acid.

Every estimation was carried out in duplicate, the agreement being good (5 %). The agreement with the manometric method was also good (difference less than 10 %), but only with amounts larger than 50  $\mu\text{l}$ .

*Fatty acids.* All the existing methods for fatty acid estimation require relatively large amounts of acid. In order to obtain greater accuracy and a shorter time of distillation we have used small volumes. Distillation was carried out after treating the samples with copper-lime reagent, because glucose can give rise to the formation of distillable acid. For estimating acids of 3 to 8 C atoms in the presence of acetic acid, we have taken advantage of the fact that the latter is not oxidized by dichromate. Interfering volatile substances were

eliminated by alkaline evaporation in the presence of  $\text{HgO}$ . Decanoic acid can also be estimated in this way, but it was not used in our experiments because of the insolubility of its Ca salt.

The details of the fatty acid estimations are as follows.

*Precipitation of proteins.* In experiments with liver slices the liquid can be directly treated with copper-lime in the amounts given by Edson [1935, 1]. For liver brei, proteins were precipitated with 1 ml. 10 %  $\text{ZnSO}_4$  per ml. brei and  $\text{NaOH}$ , the amount of which was ascertained by titrating the zinc sulphate in the presence of phenol red to an orange yellow. The liquid was then diluted 7 times and filtered. Zn cannot be used with octanoic acid as it forms an insoluble salt. Hg and Cu octanoates are also insoluble, but the latter redissolves on adding  $\text{Ca}(\text{OH})_2$  and recovery is quantitative.

Samples for acidimetric titration were directly distilled, whereas those for dichromate oxidation were treated as follows:

*Elimination of interfering substances.* The sample (6–8 ml.) after deproteinization was measured into a test tube ( $15 \times 140$  mm.), followed by 0.2 ml. 2.5  $N$   $\text{NaOH}$ , 0.2–0.3 g. powdered  $\text{HgO}$  (yellow) and a small piece of porous porcelain. It was then placed in a boiling salt water bath ( $105^\circ$ ), the test tube rack being suspended in such a way that the tubes were only partly immersed, so that their boiling could be easily controlled.

Boiling was continued until the samples were evaporated to half volume (about 1–2 hr.), the liquid being then ready for distillation.  $\text{HgO}$  has also been used by Friedemann [1938] in order to remove aldehydes, formic, pyruvic and crotonic acids, etc.

*Distillation.* An all glass apparatus similar to that described by Nicloux *et al.* [1934] was used. As it is very important that the rate of distillation should be reproducible and constant, electric heating was used, the column was covered with cotton wool and the flask surrounded by a wide glass tube.

6 ml. of the sample were measured into the distillation flask, followed by 2 g. anhydrous  $\text{Na}_2\text{SO}_4$ , 1 ml.  $\text{H}_2\text{SO}_4$  (2 vol. conc.  $\text{H}_2\text{SO}_4$  to 1 vol.  $\text{H}_2\text{O}$ ) and a capillary tube to avoid bumping. Crystallization occurs before the end of the distillation if smaller amounts of  $\text{H}_2\text{SO}_4$  are used, but not under the given conditions. Distillation takes about 15 min. and was interrupted when 5 ml. distillate had collected in a  $15 \times 140$  mm. pyrex test tube.

*Acidimetric titration.* The contents of the test tube are boiled in an open flame for 10 sec. in the presence of a small crystal of  $\text{BaCl}_2$ . This removes the  $\text{CO}_2$  and detects the presence of  $\text{H}_2\text{SO}_4$ , any trace of which would cause the estimation to be discarded. The solution is then titrated with 0.01  $N$   $\text{NaOH}$  and phenolphthalein.

*Dichromate oxidation.* Oxidizing solution: 2.45 g.  $\text{K}_2\text{Cr}_2\text{O}_7$  are dissolved in 1 l. of conc.  $\text{H}_2\text{SO}_4$  (heat until white fumes appear). 10 ml. of this solution are sufficient for oxidizing up to 2 ml. 0.01  $N$  hexanoic acid. For the same amount of octanoic acid the solution should contain double the amount of  $\text{K}_2\text{Cr}_2\text{O}_7$ .

To 5 ml. distillate contained in a test tube, 10 ml. of  $\text{K}_2\text{Cr}_2\text{O}_7$ - $\text{H}_2\text{SO}_4$  are added. The liquid is allowed to fall directly on the surface of the distillate so that immediate mixing occurs. A blank with distilled water is run at the same time. The tubes are covered with a small beaker and then immersed in a boiling water bath for 1 hr. The contents of the tubes are then quantitatively transferred into a 250 ml. Erlenmeyer flask, using about 100 ml. water. After adding 1 ml. 10 %  $\text{KI}$  the liberated  $\text{I}_2$  is titrated with 0.025  $N$   $\text{Na}_2\text{S}_2\text{O}_3$ .

*Calculation.* The ml. thiosulphate used in titrating the blank minus those used for the unknown are multiplied by  $2.5 \times 22.4$  and divided by the oxidation equivalent (Table I). This gives the amount of fatty acid in  $\mu\text{l}$ .



Table I. *Acidimetric and oxidimetric estimations of fatty acid solutions*

Acid solution ml.	Titration with 0.01 <i>N</i> NaOH (ml.)		Titration with 0.01 <i>N</i> Na <sub>2</sub> S <sub>2</sub> O <sub>8</sub> (ml.)		ml. Na <sub>2</sub> S <sub>2</sub> O <sub>8</sub> ml. NaOH
	Direct	Distilled	Direct	Distilled	
			Acetic		
1.0	0.975	0.924	—	—	—
1.0	0.969	0.935	—	—	—
2.0	1.908	1.862	—	—	—
2.0	1.910	1.797	—	—	—
			Propionic		
0.5	0.465	0.431	4.86	5.11	—
0.5	0.461	0.424	5.04	5.08	11.9
1.0	0.922	0.875	9.80	10.10	—
1.0	0.925	0.865	9.90	9.75	11.4
2.0	1.775	1.695	19.80	19.95	—
2.0	1.790	1.735	20.00	19.60	11.5
			Butyric		
0.5	0.438	0.455	8.10	8.56	—
0.5	0.443	0.473	8.09	8.32	17.3
1.0	0.918	0.932	15.93	16.10	—
1.0	0.927	0.948	15.98	15.98	17.1
2.0	1.850	1.859	31.50	31.80	—
2.0	1.854	1.870	31.55	31.20	16.9
			Valeric		
0.5	—	0.461	—	11.04	—
0.5	—	0.458	—	10.91	23.9
1.0	0.985	0.956	21.83	21.67	—
1.0	0.991	0.935	21.40	21.51	22.8
2.0	—	1.875	—	42.50	—
2.0	—	1.862	—	42.40	23.8
			Hexanoic		
0.5	—	0.430	—	11.13	—
0.5	—	0.454	—	11.40	25.6
1.0	—	0.920	—	22.65	—
1.0	—	0.938	—	22.65	24.65
2.0	—	1.830	—	43.80	—
2.0	—	1.860	—	43.20	23.6
			Heptanoic		
0.5	—	0.432	—	14.30	—
0.5	—	0.455	—	13.80	32.0
1.0	—	0.900	—	26.30	—
1.0	—	0.894	—	26.70	29.5
2.0	—	1.800	—	52.60	—
2.0	—	1.760	—	51.70	29.2
			Octanoic		
0.5	—	0.413	—	18.0	—
0.5	—	0.420	—	17.63	42.7
1.0	—	0.795	—	32.9	—
1.0	—	0.787	—	34.0	42.4
2.0	—	1.483	—	65.9	—
2.0	—	1.438	—	—	45.0

The results obtained by applying these methods to pure solutions are shown in Table I. 95 % of the acetic acid is recovered after distillation, and recovery is quantitative for the other acids within the titration error. Results of the K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> oxidation show errors not exceeding 10 %, which is satisfactory for work with liver slices. There are some differences in the oxidation equivalents, these errors being specially due to the acidimetric titration. With small amounts of acids this error becomes greater; and with higher fatty acids which are insoluble in water, the formation of the Na salt takes some time and requires strong shaking. This explains the too high value obtained in the oxidation equivalent when 2 ml. of octanoic acid were used (Table I).

*Formic acid.* Distillation under the described conditions is not quantitative (about 70 %), therefore estimations with  $\text{HgCl}_2$  were carried out on the samples after copper-lime treatment. The method was used as described by Riesser [1915] but with smaller amounts. In a test tube with a ground glass stopper, 5 ml. of the filtrate were carefully neutralized (phenol red), and 1 ml. of the  $\text{HgCl}_2$  reagent added ( $\text{HgCl}_2$  300 g., Na acetate 300 g., NaCl 80 g., per l.). The tubes were then heated in a salt water bath ( $105^\circ$ ) for 40 min. After cooling, 0.5 ml. glacial acetic acid, 1 ml. saturated KI and 2 ml. 0.03  $N$   $\text{I}_2$  were added. The tubes were shaken, and after complete solution of the calomel the excess  $\text{I}_2$  was titrated with 0.01  $N$   $\text{Na}_2\text{S}_2\text{O}_3$ .

With pure solutions the results are reproducible within 10% with amounts ranging from 30 to 300  $\mu\text{l}$ . (0.06–0.6 mg.).

*Units.* Results are given in  $\mu\text{l}$ ., the acids being considered as perfect gases at N.T.P.<sup>1</sup> (22.4  $\mu\text{l}$ . = 1  $\mu\text{mol}$ .).  $Q$  represents  $\mu\text{l}$ . of substance formed per mg. tissue (dry wt.) per hr.

#### EXPERIMENTAL RESULTS

One of the difficulties in the interpretation of the results is that in the control there is always a spontaneous formation of ketonic acids and that it is impossible to know if this continues at the same rate when a substrate is added. This also applies to the measurements of  $\text{NaHCO}_3$ . Liver slices with no substrate produce a decrease in  $\text{NaHCO}_3$ , less than half of which is due to ketonic acids. The rest is not due to lactic acid or to a distillable acid. Perhaps it is due to a fixation of base ( $K$ ) by the liver slices.

For this reason we shall often refer to the corrected  $Q$ . This is the value obtained by subtracting the value of  $Q$  given by a control with no substrate. Measuring as we have done in every case the distillable acid, acetoacetic and  $\beta$ -hydroxybutyric acids and  $\text{NaHCO}_3$ , we can get a rough idea of the formation of a non-distillable non-ketonic acid.

Slices in the presence of, e.g., Na butyrate, consume the butyrate ion and an increase in  $\text{NaHCO}_3$  occurs; ketonic acids are formed decreasing the  $\text{NaHCO}_3$ , and if any other acid is formed it will also decrease  $\text{NaHCO}_3$ . We should then have:

$$-Q_{\text{bic. (corr.)}} = Q_{\text{dist. ac.}} + Q_{\text{ketonic ac.}} + Q_{\text{NN}}$$

$Q_{\text{NN}}$  would therefore represent the non-distillable non-ketonic acid. Naturally, as this is calculated indirectly,  $Q_{\text{NN}}$  will only be significant when its value is large.

*Formic acid.* Liver slices without substrate give rise to the formation of a substance which is estimated as formic acid (see Table II, Nos. 1 and 2). This amounts to about 24  $\mu\text{l}$ . per ml., giving a  $Q_{\text{formic}}$  of 1.27 and 0.75. The method of estimation used is far from specific and we cannot assert that this substance is really formic acid.

On adding formic acid to liver slices a small disappearance occurs:  $Q_{\text{formic}} = -1.1$  and  $-0.06$ . Subtracting the spontaneous formation, the values for the disappearance ( $-Q$ ) would be 2.37 and 0.81 respectively. Ketonic acid formation is not modified and the acid disappearance is in good agreement with the changes in  $\text{NaHCO}_3$ :  $Q_{\text{bic. (corrected)}}$  2.44 and 0.65. The velocity of disappearance of formic acid is therefore small, and if it were formed from added fatty acids we should expect it to accumulate to a certain extent in the medium. As we shall see later, this is not the case.

<sup>1</sup> We have continued using  $\mu\text{l}$ . because it is the unit used by all those who have worked with tissue slices, but it would be more correct to use  $\mu\text{mol}$ .

Table II. *Liver slices from rats starved 24 hr.*Bicarbonate Ringer. Gas O<sub>2</sub> + 5 % CO<sub>2</sub>. Δ indicates the difference in composition (in μl.) of 1 ml. medium before and after 2 hr. at 37.5°

No	Volume of medium ml.	Dry wt. of slices (mg.)	Substrate	Bicarbonate		Acid by oxidation		Distillable acid		Acetoacetic acid Δ		β-Hydroxy-butyric acid		Total ketonic acids	
				Δ	Q	Δ	Q	Δ	Q	Tit.	Manom.	Δ	Q	Δ	Q
1	18.0	183	Formate 0.0118 M	-20	-0.96	-23	-1.1	—	—	25	33	21	46	+2.2	
	18.0	170	None	-64	-3.40	+24	+1.27	—	—	27	33	23	50	+2.85	
2	17.4	276	Formate 0.008 M	-26	-0.82	-2	-0.06	—	—	6	8	7	13	+0.44	
	17.4	278	None	-47	-1.47	+24	+0.75	—	—	4	9	8	12	+0.38	
3	18.8	254	Acetate 0.0136 M	+75	+2.8	—	—	-144	-5.3	12	18	20	32	+1.2	
	18.8	234	None	-45	-1.81	—	—	0	0	3	—	7	10	+0.4	
4	20.8	246	Acetate 0.0107 M	+55	+2.3	—	—	-91	-3.8	26	29	12	38	+1.7	
	20.8	219	None	-26	-1.2	—	—	0	0	7	6	16	23	+1.1	
5	20.8	228	Acetate 0.0284 M	+49	+2.2	—	—	-112	-5.1	46	51	19	65	+3.0	
	20.8	222	None	-44	-2.2	—	—	0	0	13	20	13	26	+1.2	
6	18.0	235	Propionate 0.0125 M	-15	-0.57	-58	-2.22	-46	-1.80	5	9	9	14	+0.54	
	18.0	251	None	-25	-0.90	0	0	0	0	3	4	7	9	+0.25	
7	18.0	273	Propionate 0.01 M	-32	-1.0	-27	-0.89	-9	-0.33	12	21	13	25	+0.84	
	18.0	270	None	-37	-1.23	0	0	0	0	5	11	12	16	+0.57	
8	18.0	184	Butyrate 0.0135 M	-14	-0.7	199	-9.8	-175	-8.6	100	100	56	155	+7.6	
	18.0	191	None	-26	-1.2	0	0	0	0	4	12	10	14	+0.7	
9	18.0	226	Butyrate 0.0133 M	-24	-0.9	-242	-9.7	-237	-9.5	158	157	71	229	+9.1	
	18.0	198	None	-19	-0.9	0	0	0	0	+8	+10	+10	+17	+0.5	
10	19.0	206	Butyrate 0.0177 M	-12	-0.6	-167	-7.7	-187	-8.6	119	120	65	184	+8.5	
	19.0	188	None	-29	-1.5	0	0	0	0	4	10	1	5	+0.3	
11	17.4	292	Valerate 0.0090 M	-80	-2.38	-68	-2.03	-47	-1.40	6	17	17	24	+0.71	
	17.4	245	None	-72	-2.57	0	0	0	0	4	8	13	17	+0.60	

12	17-4	244	Valerate 0-0086 <i>M</i>	-52	-1-86	-63	-2-25	-52	-1-86	19	24	19	38	+1-36
	17-4	250	None	-42	-1-46	0	0	0	0	6	10	10	16	+0-56
13	17-4	190	Hexanoate 0-0087 <i>M</i>	-97	-4-45	-164	-7-55	-137	-6-28	144	147	99	243	11-1
	17-4	195	None	-35	-1-56	0	0	0	0	24	29	18	42	1-82
14	18-0	151	Hexanoate 0-0121 <i>M</i>	-61	-3-63	-97	-5-8	-81	-4-8	102	107	39	+141	+8-4
	18-0	178	None	-43	-2-17	0	0	0	0	25	32	15	+40	+2-0
15	18-0	226	Hexanoate 0-0117 <i>M</i>	-64	-2-55	-120	-4-77	-125	-4-97	87	89	84	+170	+6-37
	18-0	233	None	-48	-1-85	0	0	0	0	4	9	10	-14	+0-54
16	17-4	201	Heptanoate 0-0095 <i>M</i>	-49	-2-12	-65	-2-81	-42	-1-86	22	28	15	38	+1-64
	17-4	228	None	-72	-2-72	0	0	0	0	22	29	11	33	+1-26
17	17-4	256	Heptanoate 0-0103 <i>M</i>	-95	-3-23	-91	-3-1	-23	-0-78	13	21	23	36	+1-22
	17-4	244	None	-56	-2-00	0	0	0	0	6	13	10	16	+0-57
18	17-4	213	Octanoate 0-01 <i>M</i>	-138	-5-6	-156	-6-4	-110	-4-5	+106	+111	+92	+198	+8-1
	17-4	175	None	-51	-2-7	0	0	0	0	4	8	4	8	0-4
19	17-4	160	Octanoate 0-0089 <i>M</i>	-174	-9-4	-131	-7-1	-88	-4-8	+112	+116	+122	+234	+12-8
	17-4	114	None	-86	-4-7	0	0	0	0	9	17	+6	+15	+0-8
20	17-4	196	Octanoate 0-0096 <i>M</i>	-116	-5-2	-155	-6-9	-96	-4-3	+144	+137	+87	+231	+10-2
	17-4	246	None	-44	-1-6	0	0	0	0	+25	+20	+14	+38	+1-3
21	17-4	234	Octanoate 0-0090 <i>M</i>	-154	-5-7	-144	-5-4	-98	-3-7	+73	+74	+165	+238	+8-9
	17-4	230	None	-55	-2-1	0	0	0	0	+5	+10	+12	+18	+0-7
22	17-5	244	Octanoate 0-008 <i>M</i>	-123	-4-42	-121	-4-34	-70	-2-51	45	49	133	178	+6-39
	17-5	269	Acetate 0-022 <i>M</i>	+121	+3-06	0	0	-205	-6-69	14	23	16	30	+0-98
23	17-5	221	Octanoate 0-0095 <i>M</i>	-94	-3-72	-117	-4-64	-88	-3-49	63	68	115	178	+7-05
	17-5	236	Acetate 0-0125 <i>M</i>	+90	+3-34	0	0	-125	-4-64	7	—	8	15	+0-56
24	17-5	237	Octanoate 0-0079 <i>M</i>	-97	-3-58	-128	-4-72	-71	-2-62	98	100	116	215	+7-87
	17-5	244	Acetate 0-0125 <i>M</i>	—	—	0	0	-134	-4-81	15	22	15	30	+1-08

*Acetic acid.* Acetic acid disappears at a rate more than twice that of formic acid.

The values of  $-Q_{\text{acetic}}$  obtained by distillation and titration with NaOH were 5.3, 3.8 and 5.1; corresponding  $Q_{\text{bic.}}$ , 4.6, 3.5 and 4.4.  $Q_{\text{keto.}}$  (corrected) amounted to 0.8, 0.6 and 1.8.

Calculating with these results the non-distillable, non-ketonic acid ( $Q_{\text{NN}} = -Q_{\text{bic.}} - Q_{\text{dist.}} - Q_{\text{keto.}}$ ) we obtain -0.1, -0.3 and -1.1. Therefore, when acetic acid disappears, there is no formation of any other acid except acetoacetic and  $\beta$ -hydroxybutyric.

It is clear from these experiments that the increase in ketonic acids only accounts for a small fraction of the acetic acid which disappears. The amount of acetic acid which disappears is 6.6, 6.3 and 2.8 times greater than the ketonic acids formed (mol. per mol.).

The mechanism of this reaction has been discussed by Krebs & Johnson [1937]. They give good evidence that the first step is a condensation of acetic with pyruvic acid, acetopyruvic acid being formed. The latter is then transformed into ketonic acids.

Acetic acid increases  $Q_{\text{O}_2}$  by 2-4 units and is therefore probably oxidized. If the oxidation were direct, the only possible intermediary would be glycollic acid, which would then be oxidized to glyoxylic and this acid might give 2 mol. of formic acid or be oxidized to oxalic acid. But this does not occur in liver, as is proved by the experiment in Table III in which the changes in bicarbonate and ketonic acids were measured.

Table III. *Liver slices in bicarbonate Ringer*

	$Q_{\text{bic.}}$	$Q_{\text{keto.}}$
No substrate	-1.30	0.34
Acetate 0.02 M	+2.69	1.38
Glycollate 0.02 M	-2.92	0.19
Oxalate 0.02 M	-1.23	0.78

This experiment shows that the acetate ion disappears, producing an increase in base ( $Q_{\text{bic.}}$ ). In the presence of glycollate this increase in base does not occur; on the contrary there is a slight acidification which might be due to oxidation to oxalate. Oxalic acid is not oxidized, for if this were the case it would give two basic equiv. per mol.

In another identical experiment formic acid was also estimated, no difference being found between the flask with no substrate and that with glycollic acid.

If acetic acid disappears by condensation with another substance one would expect that the addition of that substance would increase the rate of disappearance. Experiments in this direction were not quite satisfactory, because our method was not capable of detecting very small changes. Nevertheless we have tried many substances ( $\text{C}_4$  dicarboxylic acids, glycine, aspartic acid, insulin, dry thyroid, glucose, fructose, lactate, citrate, liver and yeast extracts etc.) without finding any appreciable increase in the rate of disappearance.

Malonic acid inhibits acetic acid disappearance ( $M/50$  malonate decreases the  $-Q_{\text{acetic}}$  from 5.1 to 2.1).

*Propionic acid.* The rate of disappearance is small ( $Q_{\text{propionic}} = -2.22$  and  $-0.89$ ); decrease in distillable acid,  $-1.80$  and  $-0.33$ .  $Q_{\text{bic.}}$  (corrected) = 0.33 and 0.23; increase in ketonic acids = 0.29 and 0.27.

As propionic acid is metabolized slowly we have not tried to determine what is the first reaction product.

*Butyric acid.* Of all the acids studied butyric is oxidized most rapidly ( $-Q_{\text{butyric}} = 9.8, 9.7$  and  $7.7$ , Table II). The corresponding values of  $Q_{\text{keto}}$  were  $6.9, 8.6$  and  $8.2$ . Therefore 70, 89 and 106 % of the butyric acid was transformed into ketonic acids.

The values of  $Q_{\text{blc}}$  (corrected) were  $0.5, 0$  and  $0.9$ . This shows that only a small amount is totally oxidized (5, 0 and 12 % respectively).

*Valeric acid.* Experiments with valeric acid (Nos. 11 and 12, Table II) gave the following results:  $Q_{\text{valeric}} = -2.03$  and  $-2.25$ .  $Q_{\text{dist. ac.}} = -1.40$  and  $-1.86$ .  $Q_{\text{blc}}$  (corrected) =  $+0.2$  and  $-0.40$ .  $Q_{\text{keto}}$  (corrected) =  $0.11$  and  $0.8$ . The difference between the values obtained by titration with NaOH and by oxidation are too small to be significant. The amount of non-distillable non-ketonic acid would be  $1.09$  and  $1.46$ ; values to which no importance can be given owing to the indirect way in which they are calculated.

*Hexanoic.* The rate of disappearance of hexanoic acid, as measured by the oxidation method, was  $-Q_{\text{hexanoic}} = 7.55, 5.8$  and  $4.77$ ; and as measured by distillation and titration with NaOH:  $-Q_{\text{dist. ac.}} = 6.28, 4.8$  and  $4.97$ . The difference between these values ( $1.27, 1.0$  and  $0$ ) is attributed to a small accumulation of acetic acid.

If we suppose that each molecule of hexanoic gives rise to one of ketonic acid and one of acetic, the  $Q_{\text{ketonic ac.}}$  should be equal to the  $Q_{\text{hexanoic}}$  plus the amount of ketonic acids which are formed from acetic acid.

The values found for the  $Q_{\text{ketonic ac.}}$  (corrected) were  $9.28, 6.4$  and  $5.83$ . They are larger than the  $Q_{\text{hexanoic}}$ , the excess being:  $1.7, 0.6$  and  $1.06$ . These values are of the order of those found for acetic acid which can increase the  $Q_{\text{ketonic ac.}}$  by 1 or 2 units. Moreover, the amount of acetic acid formed should be equal to the  $-Q_{\text{hexanoic}}$ . Of this, part accumulates in the medium ( $1.27, 1.0$  and  $0$ ) and the rest ( $6.28, 4.8$  and  $4.97$ ) would disappear. The latter values are of the order of those found for the disappearance of added acetic acid.

The values of  $-Q_{\text{blc}}$  (corrected) were  $2.89, 1.46$  and  $0.80$ . From these we can calculate the non-distillable non-ketonic acid ( $-0.11, -0.14$  and  $-0.06$ ). These small values not only show that no fixed acid is formed but also that there is a good agreement between the different methods of estimation.

*Heptanoic* (Exps. 15 & 16, Table II). Values obtained for the disappearance of heptanoic were  $-Q_{\text{heptanoic}} = 2.81$  and  $3.1$ ;  $-Q_{\text{dist. ac.}} = 1.86$  and  $0.78$ . The difference between these values ( $0.95$  and  $2.32$ ) would indicate the accumulation of a distillable acid which is not oxidized with dichromate (acetic).

The increases in the  $Q_{\text{ketonic ac.}}$  were  $0.38$  and  $0.65$ . The  $Q_{\text{blc}}$  (corrected) =  $+0.60$  and  $-1.23$ . The non-distillable non-ketonic acid would be  $0.88$  and  $1.36$ . All these results may be interpreted by the classical  $\beta$ -oxidation: 2 mol. acetic acid and 1 mol. propionic acid being formed from each mol. of heptanoic acid.

The lanthanum reaction, using the technique described for octanoic acid, was carried out in three experiments. The final sample of the flask containing slices and heptanoic acid gave a positive reaction. The reaction loses in this case some of its value because the positive result could be due to propionic acid.

*Octanoic acid.* This acid disappears at a greater rate than any of the odd numbered acids.  $-Q_{\text{octanoic}} = 6.4, 7.1, 6.9$  and  $5.4$ . The corresponding  $Q_{\text{ketonic ac.}}$  (corrected) =  $7.7, 12, 8.9$  and  $8.2$ . Therefore each mol. octanoic acid gives rise to  $1.2, 1.7, 1.3$  and  $1.5$  mol. ketonic acid (Exps. 18, 19, 20 and 21, Table II).

The amount of acetic acid formed would be ( $Q_{\text{acetic}} = Q_{\text{octanoic}} - Q_{\text{dist. ac.}}$ )  $1.9, 2.3, 2.6$  and  $1.7$ . The  $Q_{\text{blc}}$  values (corrected) were:  $-2.9, -4.7, -3.6$  and  $-3.6$ .

The calculation of the amount of non-distillable non-ketonic acid gives negative values ( $-0.3$ ,  $-2.5$ ,  $-1.0$  and  $-0.9$ ).

According to the classical  $\beta$ -oxidation each mol. octanoic acid should give one of ketonic acid and two of acetic. Therefore  $Q_{\text{ketonic}}$  should be equal to the  $-Q_{\text{octanoic}}$  and double this amount of acetic should be formed. But in our experiments the  $Q_{\text{ketonic ac.}}$  exceeds the  $Q_{\text{octanoic}}$  by 1.3, 4.9, 2.0 and 2.8. Some of these values are considerably greater than the amount of ketonic acids that arise from acetic acid and cannot be attributed to experimental errors as every estimation was carried out in duplicate. Moreover, the amount of acetic acid formed should be double the  $-Q_{\text{octanoic}}$ , that is 12.8, 14.2, 13.8 and 10.8; of this a part ( $Q \approx 2$ ) accumulates in the medium and the rest should disappear. But we have seen that acetic disappears at most at a rate of  $Q = 4-6$ . If we suppose that each mol. of octanoic acid is split into two of ketonic acid the  $Q_{\text{ketonic}}$  should be double the  $Q_{\text{octanoic}}$  and this does not explain the experimental results.

We may then suppose that octanoic acid can be oxidized by both mechanisms, a fraction ( $a$ ) would give 2 mol. ketonic acid, and the rest ( $b$ ) would give 1 mol. ketonic acid and 2 mol. acetic acid.

To test this hypothesis we carried out another set of experiments (Nos. 22, 23 and 24) in which acetate was added to the control, enabling us to ascertain for the liver specimen how much acetate disappears and the amount of ketonic acids which are formed from it.

The amount of octanoic acid which is oxidized by the mechanisms ( $a$ ) and ( $b$ ) is calculated as follows:  $Q_{\text{octanoic}} = a + b$  (I). The amount of ketonic acids formed will be (II)  $2a + b + Q_{\text{ketonic (control)}}$  (this represents the amount formed spontaneously and from acetic acid).

Replacing in (II) the value of ( $b$ ) in (I) we obtain:

$$a = Q_{\text{ketonic}} - Q_{\text{octanoic}} - Q_{\text{ketonic (control)}}.$$

On applying this equation to the experimental results we obtain the values given in Table IV.

Table IV

$-Q_{\text{octanoic}}$	$a$	$b$	$\begin{matrix} b \\ -Q_{\text{octanoic}} \end{matrix}$
4.34	1.07	3.27	0.75
4.64	1.85	2.79	0.60
4.72	2.07	2.65	0.56

We can now calculate the amount ( $2b$ ) of acetic acid formed from octanoic acid. We know by experiment how much acetic acid accumulates and we also know the rate at which acetic acid disappears in that liver (from the control). We can then compare the values for acetic acid formation calculated from the  $Q_{\text{octanoic}}$  and the  $Q_{\text{ketonic}}$  with the values obtained from the accumulated acetic acid: ( $Q_{\text{dist. ac.}} - Q_{\text{octanoic}}$ ) plus the acetic acid which disappears in the control.

The  $-Q_{\text{acetic}}$  of the control = 6.69, 4.64 and 4.81. Accumulated acetic = 1.83, 1.15 and 2.10. The sum of these values would be the acetic acid formed from octanoic = 8.5, 5.7 and 6.9 (calc. values ( $2b$ ) = 6.5, 5.6 and 5.3). The agreement is good if we consider that the acetic acid formed is calculated in an indirect way.

*Acetic acid formation from octanoic acid.* In the experiments with octanoic acid we have found a difference of 1 or 2 units in the  $Q$  as measured by oxidation and as measured by titration with NaOH. This we have attributed to an accumulation of acetic acid. These results may also be attributed to the formation of acids with less C atoms (formic, butyric or hexanoic).

Formic acid formation is excluded because estimations showed no difference between slices with and without octanoic acid. Butyric acid disappears faster than octanoic so that its accumulation is not probable.

*Lanthanum reaction.* In order to confirm the fact that acetic acid is really formed we have used the lanthanum reaction. Acetic acid does not give the lanthanum reaction in the presence of octanoic acid, which must be removed as its less soluble Ag salt before carrying out the test.

The liquid to be examined was treated with copper-lime, the alkaline filtrate was evaporated to dryness in a water bath; the residue was extracted with hot water (6 ml.) and distilled. The distillate (5 ml.) was carefully neutralized, solid  $\text{Ag}_2\text{SO}_4$  added and the solution was boiled and then cooled to  $-2^\circ$  and filtered. The filtrate was distilled and the lanthanum reaction [Krüger & Tschirch, 1930] applied to the distillate.

As in all the experiments, liver slices (200 mg. dry wt.) were suspended in 30 ml. of  $\text{NaHCO}_3$ -Ringer in two flasks. One of them contained octanoate (0.01 *M*) and the other no substrate. An initial sample of 12 ml. was withdrawn from each and the rest left 2 hr. at  $37^\circ$ . Of these four samples treated in the same manner only one gave a positive lanthanum reaction. This corresponded to the final sample of the flask with octanoate.

That acetic acid responsible for the positive lanthanum reaction does not arise from the action of alkali on acetoacetic acid was proved by adding  $\beta$ -hydroxybutyric acid to some of the controls. Although a considerable formation of acetoacetic acid occurred, the La reaction was always negative.

#### OXIDATION OF BUTYRIC ACID IN A CELL-FREE "BREI"

*Preparation of the "brei".* We have used a similar procedure to that described by Potter & Elvehjem [1936]. A bulb is blown at the end of a capillary tube in such a way that it fits with less than 0.3 mm. clearance in a strong test tube (16  $\times$  150 mm. or 22  $\times$  200 mm. according to the amount of tissue). The liver of one or two recently killed rats is weighed, cut in small portions with scissors and put in the test tube which already contains the cooled alkaline buffer (31 g.  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  + 8 g. KCl per l.: 1.53 ml. per g. liver). With the test tube in a freezing mixture, the piston is introduced and worked up and down energetically. When the contents are frozen the operation is continued out of the freezing mixture. After thawing, the tube is again put in the freezing mixture. The procedure is continued for 10 min., 0.5 *M*  $\text{KH}_2\text{PO}_4$  (0.87 ml. per 10 ml. of alkaline buffer) is added and the liquid is filtered through muslin. During all these manipulations the contents of the tube are aerated with  $\text{O}_2$ .

Microscopical examination<sup>1</sup> of the "brei" so obtained shows the absence of liver cells, only some nuclei and white and red blood cells being visible. (Blood has no oxidative activity.) The resulting pH is optimal for the preparation, addition of small amounts of acid or alkaline buffer decreasing the activity.

Activity measurements can only be made by estimating butyric acid. The  $\text{O}_2$  uptake without added substrate is so great that it cannot be used to determine the rate of butyric acid oxidation.

Butyric acid added to this "brei" at  $25^\circ$  in  $\text{O}_2$  disappears. That this disappearance is due to oxidation is proved by estimation of acetoacetic and  $\beta$ -hydroxybutyric acids. The "brei" is rapidly inactivated in absence of  $\text{O}_2$ : it is sufficient to leave it in a test tube at room temperature for 15–30 min. without bubbling  $\text{O}_2$  through it, in order to obtain complete inactivation.

<sup>1</sup> We are indebted to Dr Porto for the microscopical study of our preparations.



Succinic, fumaric, malic and citric acids added to the "brei" increase the disappearance of butyric acid but exert no action once the "brei" has been inactivated by anaerobiosis.

Table V. *Cell-free liver "brei" 7 ml. (about 700 mg. dry wt.). 2 hr. at 25°. Gas O<sub>2</sub>*

	Butyric μl.	Δ μl.	Aceto- acetic acid μl.	β-Hydroxy- butyric acid	Total ketonic acid μl.	Ketonic Butyric
Butyrate added	3029	—	—	—	—	—
"Brei" + butyrate	2370	- 742	788	420	+ 796	1.07
" + no substrate	83	—	137	412	—	—
" + butyrate + fumarate	2159	- 924	125	835	+ 640	0.69
" + fumarate (0.01 M)	74	—	5	195	—	—

In Table V we give the results of one of three exp. with fumaric acid. It shows that the "brei" with no substrate forms a certain amount of ketonic acids. When butyric is added it disappears and there is a corresponding increase in total ketonic acids. When both butyric and fumaric acids are added more butyric disappears (sometimes 50 % or more), but the ketonic acid formation does not increase so much. With butyric acid alone the relation  $\frac{\text{total ketonic}}{\text{butyric acid}}$  was 1.07 and when fumaric was also present it was only 0.69.

Moreover, when fumaric is present more of the ketonic acid appears in the reduced state. The relation  $\frac{\beta\text{-hydroxybutyric}}{\text{acetoacetic}}$  is 0.5 with butyric acid alone and 5.3 when fumaric acid is also present.

## DISCUSSION

The methods described are suitable for the type of experiments for which we have used them, and with slight modifications might be useful for other purposes. They are good for acids with 3-8 C atoms, the oxidation with dichromate being more accurate than acidimetric titration. One important point is that acetic acid does not interfere in the oxidation method.

Measurements of the rate of disappearance of saturated fatty acids show a net difference between the odd and even series. This difference is also observed in the ketogenesis, but is not clear from measurements of O<sub>2</sub> uptake: the increases in Q<sub>O<sub>2</sub></sub> [data of Edson, 1935, 1] being C<sub>1</sub>, 1.1; C<sub>2</sub>, 3.8; C<sub>3</sub>, 0.6; C<sub>4</sub>, 4.0; C<sub>5</sub>, 2.1; C<sub>6</sub>, 1.7; C<sub>7</sub>, 2.4; C<sub>8</sub>, 1.3. By measurement of the disappearance of the acids we have obtained C<sub>1</sub>, 1.5; C<sub>2</sub>, 5; C<sub>3</sub>, 2; C<sub>4</sub>, 9; C<sub>5</sub>, 2; C<sub>6</sub>, 6; C<sub>7</sub>, 3; C<sub>8</sub>, 6.

Formic acid is presumably oxidized completely to CO<sub>2</sub> and H<sub>2</sub>O. Liver slices without any added substrate give rise to a small amount of a substance which is estimated as formic acid (as was observed in liver perfusion by Toennissen & Brinkmann [1938]). Owing to the lack of specificity of the method we have used we cannot be sure if it is in fact formic acid. The formation of this substance does not increase in the presence of octanoate.

Acetic acid disappears at a much higher rate and, as bicarbonate increases proportionally, we may deduce that no other acid accumulates. Oxalic and glycollic acids do not give an increase in base; therefore they cannot be intermediaries in the disappearance; formic acid can also be excluded as its oxidation is too slow.

The amount of ketonic acids formed from acetic acid only accounts for about 20 % of that disappearing (mol. per mol.). We have not been able to find out how the rest of the acetic acid is metabolized. If it were by condensation with

another substance we might expect an increased disappearance on adding that substance but this was not observed in our experiments. The fact that there is an increase in  $O_2$  uptake with acetic acid indicates that a part of it is oxidized, although probably not directly.

The rate of disappearance of propionic acid is low and therefore we have not tried to find out how it is oxidized.

Butyric is the acid which is most rapidly oxidized by the liver. Most of it (80 or 90 %) is  $\beta$ -oxidized, but we cannot completely dismiss the possibility of a very small fraction undergoing some other type of change.

Valeric disappears at about the same rate as propionic acid. The small increase in ketonic acid formation already observed by Edson [1935,1] and Jowett & Quastel [1935,2] might arise from the acetic acid formed. Results obtained with hexanoic and heptanoic acids can be quite well interpreted by  $\beta$ -oxidation.

With octanoic acid, results are rather more complicated. This acid gives more acetoacetic +  $\beta$ -hydroxybutyric than is required by a successive  $\beta$ -oxidation, but not enough to account for the molecule breaking up into two 4 carbon units. If we suppose that a fraction of octanoic acid follows each of these possibilities, the experimental results can be well interpreted.

This type of oxidation of fatty acids has previously been suggested by Jowett & Quastel [1935, 2], although the theory was supported by somewhat indirect evidence. According to Jowett & Quastel the fatty acid molecule (e.g. octanoic) would undergo a simultaneous oxidation at the 2, 4 and 6 C atoms. The triketo-acid formed, which might only exist in combination with the enzyme, can then break down, giving 2 mol. acetoacetic or 1 mol. of acetoacetic and 2 mol. of acetic acid. This interesting hypothesis explains satisfactorily the results we have obtained. In our experiments about 30 % of the octanoic acid would be split into 2 mol. acetoacetic. Moreover, Butts *et al.* [1935] and Deuel *et al.* [1936] have found that feeding rats with measured amounts of the salts or the ethyl esters of hexanoic to tetradecanoic acids leads to the elimination of twice the expected amount of ketonic acids.

When octanoic acid is oxidized by liver slices, a certain amount of acetic acid accumulates. This was ascertained by estimation and by the lanthanum reaction.

Attempts to isolate the enzyme system which oxidizes butyric acid have failed. Precipitates obtained with acetone, ammonium sulphate and acetic acid are inactive and are not activated by adding "kochsaft" or "brei" from liver or muscle. As the inactivation is presumably due to a reduction by the substrates present in the liver, we have also investigated if the presence of oxidants would activate the preparation. However,  $H_2O_2$ , ferricyanide, iodate, quinone etc., all acted as inhibitors, and in fact nearly everything tried acted as an inhibitor.

The activating effect of dicarboxylic acids on the "brei" is difficult to understand. Szent-Györgyi [1937] has observed a similar action on the  $O_2$  uptake of pigeon muscle "brei", fumarate acting as an activator but having no action if added after a certain time.

The anaerobic inactivation of the "brei" appears in our case to be the inverse of what is known to occur with other enzymes. Thus papain, cathepsin and succinic dehydrogenase [Hopkins *et al.* 1938] are inactivated by mild oxidizing agents.

#### SUMMARY

A micromethod for the estimation of fatty acids by distillation and oxidation with dichromate is described.

The action of liver slices on normal fatty acids with 1-8 C atoms was studied.

The rates of disappearance ( $-Q$ ) of the different acids are: formic 1.5; acetic, 5; propionic, 2; butyric, 9; valeric, 2; hexanoic, 6; heptanoic, 3; octanoic, 6.

Glycollic and oxalic acids are not intermediaries in acetic acid disappearance. The ketonic acids formed only account for about 20 % of the acetic acid consumed. Butyric acid is almost completely (80–90 %) oxidized in the  $\beta$ -position.

Hexanoic and heptanoic acids also seem to follow classical  $\beta$ -oxidation.

Octanoic acid appears to be oxidized, a part giving 2 mol. of ketonic acid and another part giving 2 mol. of acetic and 1 mol. of ketonic acid. Acetic acid was identified by the lanthanum reaction.

Butyric acid oxidation can be obtained in a cell-free liver "brei". This preparation is rapidly inactivated, especially under anaerobic conditions.

C<sub>4</sub> dicarboxylic acids appear to exert an activating action on the "brei". They decrease the amount of total ketonic acids formed and increase the reduction of acetoacetic acid.

We wish to thank Prof. B. A. Houssay for his interest and advice in our work and Dr M. Dixon for kindly correcting the proofs.

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# XCV. AMMONIA CONTENT OF CANINE BLOOD AFTER ORAL ADMINISTRATION OF AMMONIUM SALTS AND AMMONIA

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*(Received 1 April 1939)*

VENULET [1934] was the first to observe that  $\text{NH}_3$  administered orally renders the blood system actually more acid. The researches of Venulet *et al.* [1936] showed that there was a fall in the alkali reserve of the blood after oral and intravenous administration of  $\text{NH}_3$ . These researches established a hitherto unexpected influence of added  $\text{NH}_3$  on the chemical composition of the blood in the living subject.

Keith & Whelan [1926] administered 100 g. of  $\text{NH}_4\text{Cl}$  intravenously to dogs, and after 2 hr. could find no changes in the ammoniacal nitrogen ( $\text{NH}_3\text{-N}$ ) in the blood. The amount of urea present simultaneously increased considerably. The removal of the liver was not followed by any increase in the quantity in the blood. In disagreement with these authors, Sasaki Takema [1936] observed an increase in the blood  $\text{NH}_3$  of dogs after intravenous injections of  $\text{NH}_4\text{Cl}$ .

Pugsley & Anderson [1934] reported enhanced diuresis and a higher blood Ca content in rats after the oral administration of 250 mg. of  $\text{NH}_4\text{Cl}$ .

Alwall *et al.* [1936] noticed that the introduction of  $\text{NH}_3$  into the stomach of rabbits evokes strong acidification but that introduction into the duodenum has absolutely no influence on the alkali reserve of the blood. As in both experiments marked symptoms of poisoning appeared, there can be no doubt that the  $\text{NH}_3$  undergoes absorption. The acidifying action of  $\text{NH}_3$  when introduced into the stomach is explained by the authors thus: the  $\text{NH}_3$  is neutralized in the stomach and enters the blood in the form of  $\text{NH}_4\text{Cl}$ . These workers hence confirm the critical remarks of Parnas [1927] with regard to Venulet's paper that the effect of the acidifying action of  $\text{NH}_3$  orally is really due to the working of the stomach.

Lazzaro [1934] reported enhanced blood  $\text{NH}_3$  in some diseases, such as hepatic cirrhosis, acute yellow atrophy of the liver, asthma, and pulmonary tuberculosis.

Galloro [1932] noted an increase in blood  $\text{NH}_3$  in a state of exhaustion, starvation and after intravenous injections of ovalbumin. Glucose administered orally to the extent of 1 g. per kg. live-weight prevents the increase in blood  $\text{NH}_3$  caused by fatigue.

The object of the present work was to investigate the influence of oral administration of ammonium salts ( $\text{NH}_4\text{Cl}$ ) and of ammonia on blood  $\text{NH}_3$  and urea.

## METHODS

The procedure of determining blood  $\text{NH}_3$  has a long history behind it. After Nencki & Zaleski's first experiments [1901] many other increasingly precise methods were published. As this progress was achieved, it was found that the values of  $\text{NH}_3$  found in the blood declined steadily. We have, in our experiments,

applied the method of determining blood ammonia elaborated by Conway [1935].

Urea was determined by the method of Conway [1933], and the normal blood urea content of canine blood was found to vary between 10 and 15 mg./100 ml.

### EXPERIMENTAL

All the experiments were made on large dogs weighing from 18 to 22 kg. After determining the normal curve of  $\text{NH}_3$  formation, the animals were given a solution of ammonium chloride containing 0.5 g.  $\text{NH}_4\text{Cl}$  per kg. live-weight. Samples of blood were taken 15 min. after the oral administration of the  $\text{NH}_4\text{Cl}$ , and then after 30, 45, 60, 75, 90 min. and so on. After each collection of blood, the curve of  $\text{NH}_3$  formation was constructed by making a number of determinations within 10 min. after the blood was shed. The blood urea content was determined simultaneously. In this way experiments were carried out on four dogs (A, B, C, D), after administering  $\text{NH}_4\text{Cl}$  and also on three dogs (F, G, H), in which ammonium hydroxide was substituted for the chloride (4 ml. of 10 %  $\text{NH}_4\text{OH}$  in 40 ml. water). The blood was collected from a neck vein through a thick hypodermic needle direct into a paraffined Erlenmeyer flask containing potassium oxalate.

### RESULTS

#### *Ammonia content of normal canine blood*

Fig. 1 shows that, as in man [Conway, 1935] and rabbit [Conway & Cooke, 1938], the  $\text{NH}_3$  content of the circulating blood of the dog is either zero or below the analytical level. In canine blood we have the same characteristic rise

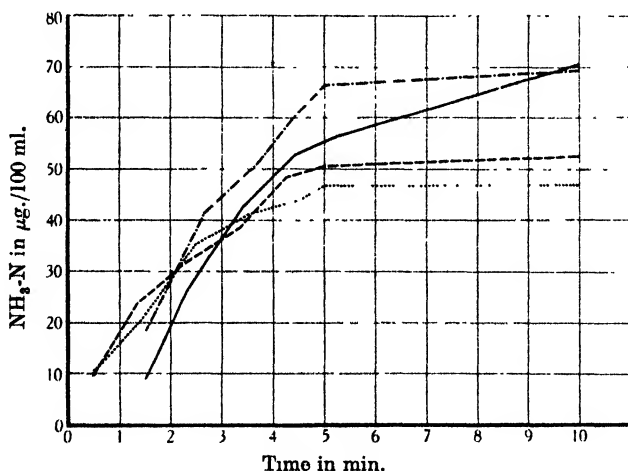


Fig. 1. Ammonia content of canine blood after shedding. — Dog A. --- Dog B. .... Dog C. —·— Dog D.

immediately after shedding and ending within 5 min., the  $\text{NH}_3$  being then formed very slowly. The curve of this  $\text{NH}_3$  formation, extrapolated to zero time after shedding, passes through the origin. The only difference between the canine and human blood, with respect to  $\text{NH}_3$  content and formation, is that the  $\text{NH}_3$  content of the former is somewhat higher at the fifth minute, being 50–70  $\mu\text{g.}$   $\text{NH}_3\text{-N}/100$  ml. instead of 40–50  $\mu\text{g.}/100$  ml. for the latter.

*Ammonia content of canine blood after oral administration of  $\text{NH}_4\text{Cl}$  (0.5 g./kg.)*

The results for the four dogs are summarized in Table I. The table gives the mean values of the blood  $\text{NH}_3$  at regular 15 min. periods after administration. The blood  $\text{NH}_3$  figures refer to the analyses conducted immediately after shedding, and after 5 and 10 min. respectively. For the 60 min. period after administration, observations for only 3 animals (B, C, D), were available.

Table I

Time after administration of $\text{NH}_4\text{Cl}$ , min.	Mean blood $\text{NH}_3$ content ( $\mu\text{g. N/100 ml.}$ ) analysed after shedding			Observed formation of $\text{NH}_3$ in shed blood $\mu\text{g. N/100 ml.}$	
	Immediately after	5 min. after	10 min. after	After 5 min.	After 10 min.
0	11.9 (0.7)	55.1	59.8	43.2	47.9
15	1013.0 (0.6)	1132	1107	119	94
30	605.0 (0.9)	749	627	144	22
45	296.0 (1.0)	320	319	24	23
60	64.0 (1.0)	114	151	50	87
75	48.0 (0.9)	79	82	31	34
90	8.0 (0.8)	43	48	35	40

The bracketed figures give the mean time in minutes after shedding.

The peak of the rise in blood  $\text{NH}_3$  must occur at some time less than 15 min., at which time there is a mean value of  $1013 \mu\text{g. NH}_3\text{-N/100 ml.}$ , as determined 0.6 min. after shedding. 90 min. after administration the blood  $\text{NH}_3$  content has again returned to zero.

*The "alpha" rise at high blood ammonia concentrations*

The immediate rise in the blood ammonia content after shedding and terminating within 5 min. has been termed the "alpha" ammonia [Conway & Cooke, 1938], to distinguish it from other phases in the curve of  $\text{NH}_3$  formation. The present experiments give an opportunity of finding how this formation is influenced by high initial blood  $\text{NH}_3$  concentrations. Table I again summarizes the necessary data, the last two columns giving the increase at the 5 and 10 min. periods over the concentrations obtained immediately after shedding. It appears from the table that such an increase in the blood  $\text{NH}_3$  occurs at all levels—as judged from the mean values—and that it comes to an end after 5 min. The amount of this formation is not influenced in any definite way by the initial height of the blood  $\text{NH}_3$ . As seen from the last column of Table I it varies as the blood  $\text{NH}_3$  changes but in no definite relation thereto. At the highest concentrations observed the experimental error alone will cause marked variations. At

Table II. *Blood collected 15 min. after oral administration of  $\text{NH}_4\text{Cl}$* 

Time range after shedding min.	Blood $\text{NH}_3$ content as $\mu\text{g. N/100 ml.}$			
	Dog A	Dog B	Dog C	Dog D
0.5	—	676.5	952.8	—
0.75	1498	—	—	923.8
1.1	—	550.2	—	—
1.5	—	—	—	826.0
2.0	1305	559.2	1206.9	—
2.5	—	—	—	859.2
3.0	1205	—	—	—
3.5	—	—	1302.0	1038.2
4.0	1246	523.2	—	—
5.0	1498	506.2	1427.7	1096.8
7.5	—	—	—	988.6
10.0	1426	676.5	1330.6	—

these highest levels also, there appears to be a greater fluctuation in the blood  $\text{NH}_3$  in the 10 min. period after shedding than can be attributed to analytical errors. Table II illustrates this. In the blood from two animals there is actually a decrease over the first 5 min., and for one blood an unduly large increase. That such effects cannot be altogether attributed to analytical errors—which will here no doubt play some part—is shown by the close agreement or uniform change of the results in Table III, for blood samples taken 45 min. after  $\text{NH}_4\text{Cl}$  administration.

Table III. *Blood collected 45 min. after oral administration of  $\text{NH}_4\text{Cl}$*

Time range after shedding min.	Blood $\text{NH}_3$ content ( $\mu\text{g. N/100 ml.}$ )			
	Dog A	Dog B	Dog C	Dog D
0.5	—	—	274.3	—
0.75	—	—	—	297.4
1.25	—	282.4	—	311.8
1.5	331.2	—	289.0	—
1.75	—	289.1	—	—
2.25	—	294.0	—	326.0
2.5	328.8	—	291.6	—
3.0	326.4	302.1	—	—
3.5	—	—	300.2	329.1
4.5	—	306.9	306.0	343.4
5.0	316.8	309.3	308.8	345.3
10.0	310.8	309.3	311.8	345.3

*Ammonia content of blood after oral administration of  $\text{NH}_4\text{OH}$*

Here the data from three dogs (F, G, H) are available. These indicate a rather marked difference from the figures after  $\text{NH}_4\text{Cl}$  administration. The blood  $\text{NH}_3$  falls with great rapidity, so that—as shown in Table IV (*vide* also Fig. 3)—it returns to normal level 45 min. afterwards. Within the interval of 15–45 min. it has fallen from about  $300 \mu\text{g. NH}_3\text{-N/100 ml.}$  to zero.

Table IV

Time after administration of $\text{NH}_4\text{OH}$ min.	Mean blood $\text{NH}_3$ content ( $\mu\text{g. N/100 ml.}$ ) analysed after shedding			Observed formation of $\text{NH}_3$ in shed blood $\mu\text{g. N/100 ml.}$	
	Immediately after	5 min. after	10 min. after	After 5 min.	After 10 min.
0	9.9 (0.8)	63.6	66.3	53.7	56.4
15	305.4 (0.6)	365.6	367.0	60.2	61.6
30	199.5 (0.8)	266.1	267.7	66.6	68.2
45	3.2 (1.0)	56.7	58.8	53.5	55.6

The bracketed figures give the mean time in minutes after shedding.

*The blood urea content after oral administration of  $\text{NH}_4\text{Cl}$  and  $\text{NH}_4\text{OH}$*

Blood urea analyses were carried out for the same dogs and in the same experiments summarized in Tables I and IV. The data are given in Tables V and VI. In Figs. 2 and 3 the mean blood urea changes are compared with those of the blood  $\text{NH}_3$  after  $\text{NH}_4\text{Cl}$  and  $\text{NH}_4\text{OH}$  administration. To facilitate the graphical comparison, the blood urea-N in Fig. 2 is given in units 10 times greater than that of the blood  $\text{NH}_3$ .

It is clear from Tables V and VI and Figs. 2 and 3 that a marked increase of the blood urea occurs when the blood  $\text{NH}_3$  has returned to zero, the significance of which is discussed below. The great reactivity of the liver to blood  $\text{NH}_3$ , resulting in the formation of urea is also manifest.

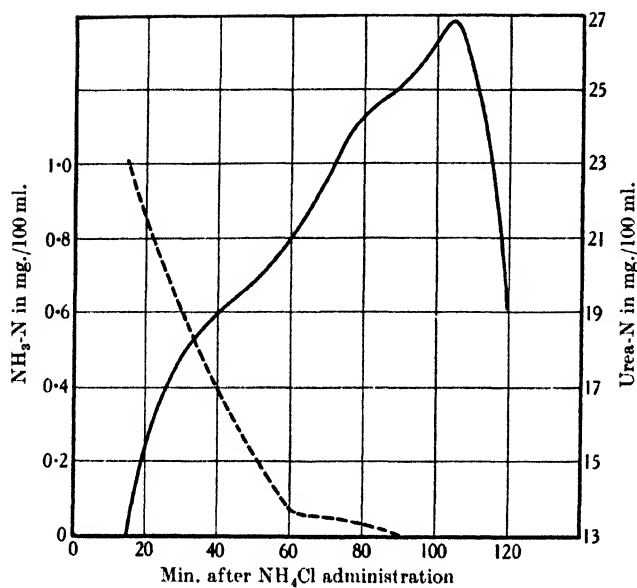


Fig. 2.

Fig. 2. Mean curves of blood  $\text{NH}_3$  and blood urea for four dogs after oral administration of  $\text{NH}_4\text{Cl}$ . — Blood urea. - - - Blood ammonia.

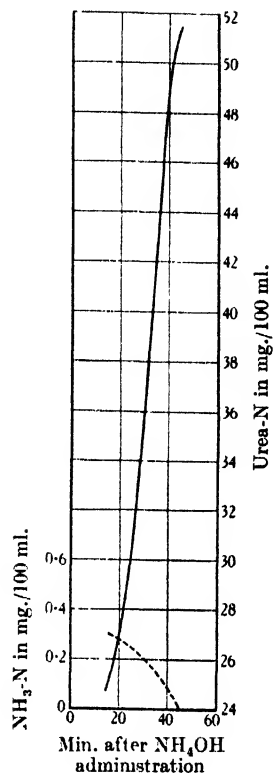


Fig. 3.

Fig. 3. Mean curves of blood  $\text{NH}_3$  and blood urea for three dogs after oral administration of  $\text{NH}_4\text{OH}$ . — Blood urea. - - - Blood ammonia.

Table V. *Urea determinations*

Time after oral administration of $\text{NH}_4\text{Cl}$ min.	Blood urea content (mg. urea-N/100 ml.)			
	Dog A	Dog B	Dog C	Dog D
0				
15	10.1	—	15.4	13.6
25	—	26.0	—	—
30	12.1	—	18.1	15.6
45	12.2	21.7	24.2	—
60	—	23.6	24.6	15.8
75	15.4	27.9	28.0	23.5
90	18.4	27.0	33.1	21.4
105	—	27.0	33.3	20.5
120	14.4	—	27.2	15.8

Table VI. *Urea determinations*

Time after oral administration of $\text{NH}_4\text{OH}$ min.	Blood urea content (mg. urea-N/100 ml.)		
	Dog F	Dog G	Dog H
0	14.2	18.6	13.3
15	14.2	41.0	18.6
30	48.4	43.0	18.0
45	51.3	50.0	53.1



## DISCUSSION

When we consider the rapidity with which the blood  $\text{NH}_3$  falls from a very rapidly attained peak value after administration of ammonium salts or  $\text{NH}_3$ , it is not surprising that the normal content of  $\text{NH}_3$  in circulating blood is either zero or below the analytical level. After administration of ammonium hydroxide the blood  $\text{NH}_3$  drops from approximately  $300\text{ }\mu\text{g. NH}_3\text{-N/100 ml.}$  to zero within 30 min., and during this time also (15–45 min. after administration) considerable amounts of ammonia are doubtless still entering from the intestine.

Only a very small part of such decrease can be attributed to excretion by the kidneys, and probably the fall is mostly accounted for by the activity of the liver.

The blood urea rises to a considerable height when compared with the blood  $\text{NH}_3$ , and it is noteworthy that the most rapid increase generally occurs when the blood ammonia has returned again almost to the zero level with a marked increase continuing when this level is reached. This indicates a considerable lag between the initial stage of the fixation of the  $\text{NH}_3$  by the liver and its final appearance as urea. From what we know of the stages of urea formation [Krebs, 1932] the first stage would represent the formation of citrulline from ornithine,  $\text{NH}_3$  and  $\text{CO}_2$ , the succeeding stages—if we may judge from these experiments—proceeding comparatively slowly. It may be inferred that the ornithine content of blood or liver will be a determining factor in the rapid removal of traces of  $\text{NH}_3$  from the blood stream, and will explain why in the fasting animal an increase in the blood  $\text{NH}_3$  has been observed [e.g. Parnas & Wagner, 1924].

The immediate or "alpha" rise in the blood  $\text{NH}_3$  after shedding is apparently independent of the absolute value of the free blood  $\text{NH}_3$ , which is only to be expected if it derives from the deamination of some blood substance. At the same time the analyses at the highest blood  $\text{NH}_3$  concentrations observed indicate an interplay between some fixation and some formation of the free  $\text{NH}_3$ .

## SUMMARY

1. The normal blood  $\text{NH}_3$  content for resting dogs is either zero or below the analytical level, when determined by the method of Conway. There appears also the same characteristic rise immediately after shedding and ending within 5 min., as described for the human subject [Conway, 1935].

2. Oral administration of  $\text{NH}_4\text{Cl}$  (0.5 g./kg.) causes a marked increase in the blood  $\text{NH}_3$ , the highest mean value of approximately 1.0 mg./100 ml. being observed 15 min. after administration and the peak probably occurring in less than this time. The return to normal occurred within 90 min.

3. When the blood  $\text{NH}_3$  is so increased there is also an increase in the blood urea of 2–36 mg./100 ml. over 90 min. The steepest rise in the blood urea occurred from 60 to 90 min. after administration of  $\text{NH}_4\text{Cl}$ , and about 45 min. after administration of  $\text{NH}_4\text{OH}$ , when the blood  $\text{NH}_3$  had returned almost to zero, indicating a marked lag in the urea formation after an initial  $\text{NH}_3$  fixation.

4. Oral administration of  $\text{NH}_4\text{OH}$  (4 ml. of 10 %  $\text{NH}_4\text{OH}$  in 40 ml. water) is followed by a moderate rise in the blood  $\text{NH}_3$ . In 15 min. it reached 0.3 mg./100 ml. but returned to normal in the next 30 min.

Our thanks are due to Prof. E. J. Conway for his kindness in demonstrating his method to one of us, also to Prof. F. Venulet and Dr F. Goebel for their unfailing assistance and interest in this research.

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# XCVI. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN. I<sup>1</sup>

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*(Received 25 March 1938)*

WHEN a solution of pyridine-haemochromogen containing ascorbic acid is shaken in air, both substances undergo a coupled oxidation leading to verdohaemochromogen and dehydroascorbic acid [Karrer *et al.* 1933; Lemberg *et al.* 1938, 1, 2; cf. also Haurowitz,<sup>3</sup> 1937]. When haemoglobin and ascorbic acid are incubated in air at pH 7.2 and 38° a slower oxidation takes place giving choleglobin, a bile pigment-haemoglobin compound [Lemberg *et al.* 1938, 3] and finally products in which the protein is denatured [Anderson & Hart, 1934; Edlbacher & v. Segesser, 1937]. The ascorbic acid is broken down to dehydroascorbic acid, and finally to other products. Here we present our investigations of the reaction in its early stages when the main breakdown product of the haemoglobin is choleglobin.

There are two possibilities for the mechanism of this reaction.

(1) *The autoxidation of the ascorbic acid is independent of the haemoglobin.* The hydrogen peroxide formed in this reaction oxidizes the porphyrin ring to the bile pigment prosthetic group of choleglobin. This mechanism has been assumed by Barkan & Schales [1938, 1, 2, 3]. Hydrogen peroxide, indeed, causes the oxidation of the porphyrin nucleus of pyridine-haemochromogen [Lemberg *et al.* 1938, 2].

(2) *Ascorbic acid and oxyhaemoglobin react directly.* Two hydrogen atoms are transferred from ascorbic acid to oxyhaemoglobin, giving rise to an unstable haemoglobin-hydrogen peroxide compound which breaks down with the formation of choleglobin. Such a mechanism was suggested by Lemberg *et al.* [1938, 2], because the first possibility could be excluded in the aerobic experiments with pyridine-haemochromogen, the ascorbic acid not undergoing a sufficiently rapid autoxidation in the pyridine solutions used.

Under the conditions of the reaction with haemoglobin the autoxidation is faster. The phosphate buffers, ranging from pH 7.2 to 8.6, were not copper-free, and in the course of autoxidation hydrogen peroxide is formed and can be detected by the luminol test. If it can be shown, however, that the oxidation of haemoglobin proceeds under conditions in which the autoxidation of the ascorbic acid is prevented, then the first theory cannot hold and the second alone explains the facts.

The autoxidation of ascorbic is no true autoxidation, but is catalysed by copper, and we can investigate the reaction by the use of copper-inhibitors.

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<sup>2</sup> Aided by a grant from the T. E. Rofe bequest.

<sup>3</sup> The absorption band in the red observed by Haurowitz, p. 142, is not caused by substances related to chlorophylls or chlorins, but by verdohaemochromogen.

## EXPERIMENTAL

The reaction was studied spectroscopically and by measurement of the  $O_2$ -uptake. Choleglobin may be distinguished from other haemoglobin derivatives with an absorption band in the red by its behaviour with CO after reduction. The following table shows its differences from methaemoglobin, sulphaemoglobin and pseudomethaemoglobin [Fairley & Bromfield, 1937].

Compound	Absorption band in $m\mu$	With $Na_2S_2O_4$	$Na_2S_2O_4$ and CO	$Na_2S_2O_4$ and alkali
Methaemoglobin	630-634	Reduced Hb	CO-Hb	Protohaemochromogen
<i>pseudo</i> Met-Hb	622-624	Protohaemochromogen	CO-haemochromogen	Protohaemochromogen
Sulph-Hb	618-622	No change	Band at 614	Protohaemochromogen
Choleglobin	628-632	No change	Band at 627-629	Cholehaemochromogen, band at 618-620

The  $O_2$ -uptake from a solution of oxyhaemoglobin and ascorbic acid, shaken in air, was measured by the Warburg technique with KOH in the central cup. A solution of crystalline horse haemoglobin was placed in the main chamber of the vessels, while a solution of 1 mg. crystalline B.D.H. ascorbic acid was added from the side-bulb. All the solutions were buffered with  $M/15$  phosphate buffer, in the majority of experiments at  $pH$  7.4. The temperature of the bath was  $38^\circ$ .

*Inhibition of the autoxidation*

Of the copper-inhibitors used by other workers compounds of the diethyldithiocarbamate type proved best for our purpose. We used the above compound as well as piperidine-dithiocarbamate which was found to be very satisfactory. These were put in the main vessel with the buffered haemoglobin solution.

It can be seen from Fig. 1 that the inhibitor is able to prevent the autoxidation of the ascorbic acid at the  $pH$  used (curves 1 and 2). Control experiments without ascorbic acid show that the small uptake observed in the presence of the inhibitor is due to the oxidation of the inhibitor itself. The presence of the inhibitor does not affect the haemoglobin breakdown; control experiments showed that addition of the inhibitor does not increase the  $O_2$ -uptake of haemoglobin solutions any more than is due to autoxidation of the inhibitor. While the inhibitor suppresses the autoxidation of the ascorbic acid, it does not diminish its reaction with haemoglobin. Choleglobin was found to be present at the end of the experiment with inhibitor in no less strength than in experiments without inhibitor. The curves 3 and 4 of Fig. 1 show that the  $O_2$ -uptake is not diminished but even slightly increased by the copper-inhibitor. It can also be seen from these curves that dehydroascorbic acid is further oxidized. There is, in the experiment with inhibitor, a distinct diminution of the rate of  $O_2$ -uptake after some what more than one oxygen atom per mol. of ascorbic acid has been taken up, while the oxidation in all the other experiments proceeds with unaltered rate beyond this point. Experiments in which dehydroascorbic acid was used in

place of ascorbic acid showed that it was not able to form choleglobin from haemoglobin. The dehydroascorbic acid was prepared by oxidizing ascorbic acid with the calculated amount of iodine.

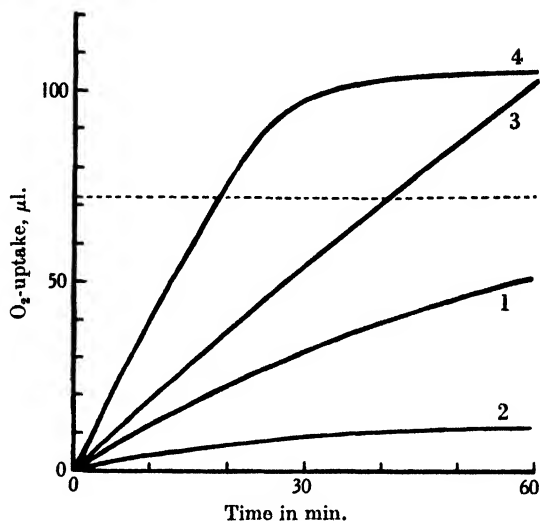


Fig. 1.  $O_2$ -uptake in Warburg manometer. The horizontal dotted line represents oxygen required to oxidize ascorbic to dehydroascorbic acid. (Curve 1, autoxidation of ascorbic acid in phosphate buffer, pH 7.4. Curve 2, the same as 1 with copper-inhibitor (1 mg.). Curve 3, coupled oxidation (0.4 ml. strong haemoglobin solution and acid in phosphate buffer, pH 7.4). Curve 4, the same as 3 with copper-inhibitor.

#### *Choleglobin formed with hydrogen peroxide and ascorbic acid*

If the haemoglobin and the ascorbic acid are put into a Thunberg tube, and the haemoglobin reduced by evacuation and the use of oxygen-free nitrogen, no reaction takes place. If hydrogen peroxide is now added through the outlet tube, a formation of choleglobin can be observed at  $38^\circ$ . This reaction takes place more rapidly than the choleglobin formation when the same quantities of haemoglobin and ascorbic acid are incubated in air.

Barkan has investigated the formation of choleglobin by the action of hydrogen peroxide on haemoglobin in the presence of cyanide. He concludes that cyanide is necessary for the formation of these bile pigment compounds, and that it cannot be replaced by reducing substances. We find that ascorbic acid can be used instead of cyanide, but we are unable at present to explain satisfactorily the action of cyanide in bile pigment-haemoglobin formation. The mechanism of the two reactions appears to be different. Barkan has withdrawn his earlier explanation that the cyanide only inhibits the catalase that is present; in this we agree.

#### *Carbon monoxide as inhibitor*

While we have thus succeeded in blocking the autoxidation without preventing choleglobin formation, we can show that the formation of the choleglobin is prevented by CO without considerably diminishing the rate of the autoxidation.

Barron *et al.* [1936] have shown that CO inhibits the autoxidation of ascorbic acid. The partial pressure of the CO in our experiments was not sufficient to do this to a great extent. Ascorbic acid and haemoglobin were allowed to react in

phosphate buffer at pH 7.4 under a gas mixture of 20 % O<sub>2</sub> and 80 % CO. The concentration of the ascorbic acid was measured by precipitating a measured volume of the contents of the vessel *in vacuo* with metaphosphoric acid and titrating the filtrate with 2:6-dichlorophenolindophenol [Lemberg & Legge, 1938].

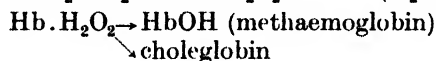
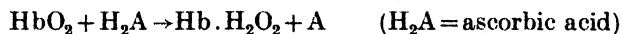
After 2 hr. at 38° nearly all the ascorbic acid had been oxidized, but no trace of choleglobin had been formed. Experiments carried out in air under the same conditions resulted in a copious formation of choleglobin. CO ought, indeed, to prevent the reaction, if the initial step consists in a direct reaction of oxyhaemoglobin with ascorbic acid.

CO does not prevent the formation of choleglobin from haemoglobin by hydrogen peroxide. Washed red cells of sheep were laked by freezing. 1 ml. of this strong oxyhaemoglobin solution was buffered with 6.5 ml. of *M*/15 phosphate buffer pH 7.4. Of this solution 2 ml. were placed in the bottom part of a Thunberg tube, the top part of which contained 0.1 ml. of 0.5 % H<sub>2</sub>O<sub>2</sub>. 2.5 mg. of ascorbic acid were added to the haemoglobin solution and the tube immediately evacuated and filled with oxygen-free CO; 5 min. after the addition of the peroxide a spectroscopic investigation revealed a distinct band at 630 mμ which persisted on reduction by hydrosulphite.

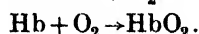
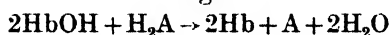
While hydrogen peroxide could be detected in the autoxidation of the ascorbic acid by the luminol test, its application to the reaction catalysed by haemoglobin gave negative results. The test was also made according to the method of Holtz & Triem [1937], whereby the reaction mixture was acidified with H<sub>2</sub>SO<sub>4</sub>, distilled *in vacuo* at 60°, and the distillate tested with luminol. The test was again negative. This is not, however, definite evidence that hydrogen peroxide is absent, because large amounts of haemoglobin or haematin suppress the chemiluminescence.

### DISCUSSION

We have brought forward evidence that the formation of choleglobin from haemoglobin in the coupled oxidation with ascorbic acid is not due to the formation of hydrogen peroxide by autoxidation of the ascorbic acid as Barkan has assumed. This reaction can be prevented without stopping the choleglobin formation. We express the formation of the choleglobin in the following way:



↘ choleglobin



The whole cycle continues until all the reducing agent is oxidized.

This process is very similar to the reaction of pyridine-haemochromogen and ascorbic acid to form verdohaemochromogen [Lemberg *et al.* 1938, 2], yet we can see the specific influence of the globin on the course of the oxidation in the different end-products. The prosthetic group of choleglobin is similar to, but not identical with, verdohaem. We are not able at present to say wherein lies the structural difference between cholehaem and verdohaem.

Barkan uses the collective prefix *pseudohaem* for compounds which may contain either cholehaem or verdohaem and makes no distinction between them. The use of the terms "*pseudohaemochromogen*" for verdohaemochromogen and "*pseudohaemoglobin*" for both choleglobin and cholehaemochromogen makes it appear that the difference between them is only one of the protein moieties of

the compounds, while in fact the main difference lies in the structure of the prosthetic group. Again, the name "*pseudohaemoglobin*" is used by Barkan for the compound with easily detachable iron in the erythrocytes. Its structure is still unknown, but it differs from choleglobin in spectroscopic features [Lemberg *et al.* 1938, 3] and in that carbon monoxide prevents the detachment of iron from it. We cannot, for that reason, conform to Barkan's nomenclature. Fairley has also used the name pseudomethaemoglobin for a quite different class of compounds.

The claim of Barkan & Schales [1938, 3] to have proved the existence of bile pigment-haemoglobin before the experiments of Lemberg *et al.* [1938, 3] cannot be accepted. The fact that iron is easily detached from compounds of this type, which in itself is based on experiments of Lemberg [1935], cannot be considered as sufficient proof of the bile pigment nature of these compounds. Only the isolation of biliverdin from choleglobin has proved that it is a bile pigment-haemoglobin [Lemberg *et al.* 1938, 3]. Full description of the properties of choleglobin will be published later.

Michel [1938] has recently suggested a similar mechanism for the formation of sulphaemoglobin from oxyhaemoglobin and hydrogen sulphide, although here, as in our experiments, hydrogen peroxide can cause the same reaction. In the presence of hydrogen sulphide, however, the product is different from choleglobin, the sulphur atom entering the haemoglobin molecule. In a forthcoming paper we shall confirm and extend Michel's evidence that sulphaemoglobin can be reconverted into protohaemochromogen and thus differs from the bile pigment-haematin compounds, to which Barkan had assumed it to belong. We can, however, confirm Barkan's finding that the iron of sulphaemoglobin can be easily detached. We have here an example of a compound with easily detachable iron which cannot be a bile pigment-haematin compound.

#### SUMMARY

The coupled oxidation between oxyhaemoglobin and ascorbic acid, leading to a bile pigment-haemoglobin, is not caused by the hydrogen peroxide liberated in the autoxidation of the ascorbic acid. This autoxidation can be prevented by copper-inhibitors without stopping the formation of choleglobin. Oxyhaemoglobin and ascorbic acid react directly to form choleglobin.

While carbon monoxide prevents the formation of choleglobin by the action of atmospheric oxygen on haemoglobin in the presence of ascorbic acid, it does not prevent the formation of choleglobin by hydrogen peroxide.

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# XCVII. PYRUVATE OXIDATION IN BRAIN

## V. EVIDENCE DERIVED FROM THE METABOLISM OF $\alpha$ -KETOBUTYRIC ACID

BY CYRIL LONG AND RUDOLPH ALBERT PETERS

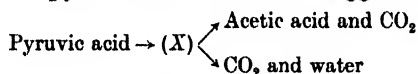
*From the Department of Biochemistry, Oxford*

*(Received 31 March 1939)*

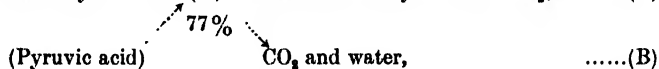
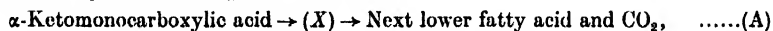
THE problem of the specificity of the pyruvate oxidation system in pigeon's brain tissue has been studied more fully by us, not only in the hope that it would throw fresh light upon the path of complete oxidation of pyruvic acid, but also in the belief that knowledge of the behaviour of other simple  $\alpha$ -keto-acids would contribute further to the elucidation of the metabolism of some of the oxidation products of the  $\alpha$ -amino-acids.

In particular it was hoped that some definite indication would be obtained as to whether the three reactions found to account quantitatively for the aerobic oxidation of pyruvic acid [Long, 1938] were entirely separate or were inter-related. This objective, we think, has been realized. We have now obtained evidence that the rate of oxidation ( $\mu$  mol./hr.) is the same for pyruvic acid and  $\alpha$ -ketobutyric acid; but whereas the latter gives as end-product  $\text{CO}_2$  and presumably the next lower fatty acid, a change requiring  $\frac{1}{2}\text{O}_2$  per mol., part of the pyruvic acid disappearing is much more fully oxidized. This suggests that there is a common path for the initial stage in the oxidation, intermediate compounds of similar type being formed from pyruvic and  $\alpha$ -ketobutyric acids, the sole difference being that in the case of pyruvic acid a further system intervenes capable of oxidizing this unstable intermediate to  $\text{CO}_2$  and water. The intermediate formed from  $\alpha$ -ketobutyric acid can only break down without  $\text{O}_2$  uptake to  $\text{CO}_2$  and, by analogy, propionic acid.

Hence the scheme for pyruvic acid oxidation suggested by Long [1938]



may be replaced by the more general scheme



with the special system for pyruvic acid. The figure 77% is given by

$$\frac{\text{Pyruvic acid giving } \text{CO}_2 \text{ and water}}{\text{Total pyruvic acid oxidized}} \times 100 = \frac{67}{67 + 19.6} \times 100 = 77,$$

no account being taken of the dismutation. This process has been shown to take place more rapidly under anaerobic conditions than in the presence of molecular  $\text{O}_2$ .

### HISTORICAL

Decarboxylation by yeast of  $\alpha$ -keto-acids, containing at least one  $\beta$ -hydrogen atom, was well established by Neuberg and his co-workers [Neuberg & Kerb, 1912; Neuberg & Peterson, 1914; also Hofmann, 1931]; it is also now known



that vitamin B<sub>1</sub> pyrophosphoric ester (cocarboxylase) is required in this system both for pyruvic acid [Lohmann & Schuster, 1937] and for  $\alpha$ -ketobutyric acid [Peters, 1937]. Contrary to its behaviour with yeast carboxylase,  $\alpha$ -ketobutyric acid showed no apparent reaction with the pyruvate oxidation system in unwashed brain and even appeared to inhibit pyruvate respiration. This provided no foothold for a belief in the similarity of the brain and yeast systems, nor did cocarboxylase prove nearly as efficient as vitamin B<sub>1</sub> in the catatorulin test [Peters, 1937]. At the same time McGowan & Peters [1937] had found no evidence for the oxidation of the  $\alpha$ -ketodicarboxylic acids,  $\alpha$ -ketoadipic and  $\alpha$ -ketoglutaric acids, by brain brei.

We have reinvestigated with yeast and brain the behaviour of  $\alpha$ -ketobutyric acid and also studied the homologue  $\alpha$ -ketovaleric acid.

## EXPERIMENTAL

### Reagents

*Pyruvic acid.* Pure Na pyruvate [Peters, 1938] was used. Before each experiment a small quantity was dissolved in Ringer phosphate, pH 7.3, so that 0.2 ml. when added to the respiration medium of total vol. 2.8 ml. gave a final concentration of c. 0.02 *M*.

*Succinic acid.* A "Kahlbaum" specimen of Na succinate was used, final concentration c. 0.04 *M*.

*$\alpha$ -Ketobutyric acid.* A sample of Na  $\alpha$ -ketobutyrate was prepared from the acid [Neuberg & Kerb, 1912] by the method of Peters [1937].

*$\alpha$ -Ketovaleric acid.* Two samples were prepared, one by Mr L. A. Stocken in this Department, by condensing together ethyl oxalate and ethyl *n*-butyrate, followed by the hydrolysis of the resulting ethyl ethyloxaloacetate with 5% H<sub>2</sub>SO<sub>4</sub>. B.P. 78°/14 mm. Low-melting colourless solid. Na salt prepared as for Na  $\alpha$ -ketobutyrate. Analysis: found: C, 43.5%; H, 5.2%. Calc. for C<sub>5</sub>H<sub>7</sub>O<sub>3</sub>Na: C, 43.5%; H, 5.1%. 2:4-Dinitrophenylhydrazone, m.p. 135–136° (corr.).

Unless otherwise stated, the concentration of  $\alpha$ -keto-acid was c. 0.02 *M*. This gave the maximal effect (cf. Tables IV, XIII).

*Vitamin B<sub>1</sub>.* Synthetic specimens from Messrs Hoffmann la Roche and Messrs Bayer.

*Cocarboxylase.* A sample (50% purity) containing a little vitamin B<sub>1</sub> prepared by Mr H. W. Kinnersley in this Department by the method of Kinnersley & Peters [1938]. Also a synthetic specimen from Messrs Merck.

Values quoted in experiments are the average of duplicates or triplicates, the variation being not greater than  $\pm 1.5\%$ .

## DECARBOXYLATION BY YEAST

In the experiments of Neuberg and his co-workers,  $\alpha$ -ketobutyric acid [Neuberg & Kerb, 1912] and  $\alpha$ -ketovaleric acid [Hofmann, 1931] were easily decarboxylated by baker's yeast, washed with phosphate buffer, pH 6.2. Peters [1937] used yeast washed with alkaline phosphate by the method of Lohmann & Schuster [1937] to show the necessity for cocarboxylase in the decarboxylation of  $\alpha$ -ketobutyric acid. Ochoa [1938] demonstrated the activation of cocarboxylase by vitamin B<sub>1</sub> in the decarboxylation of pyruvic acid. These new developments have been extended to  $\alpha$ -ketobutyric and  $\alpha$ -ketovaleric acids.

Dry baker's yeast (supplied by the Distillers Co. Ltd.) was washed free from cocarboxylase [Ochoa & Peters, 1938, 1]. CO<sub>2</sub> production was measured in air

at 28° and pH 6.2, using Barcroft-Dixon manometers. Each bottle contained 1.0 ml. alkaline-washed yeast suspension, 0.10 ml.  $\text{MgCl}_2$  (equivalent to 100  $\mu\text{g}$ . Mg), cocarboxylase and vitamin  $\text{B}_1$  where used. The pyruvic or  $\alpha$ -keto-acid was contained in a Keilin cup, tipped in after 15 min. incubation period. Table I shows that the  $\alpha$ -keto-acids are decarboxylated at about the same rate as pyruvic acid, and that the activation of cocarboxylase by vitamin  $\text{B}_1$  takes place to approximately the same extent in all cases. The yeast decarboxylation system is therefore quite general for  $\alpha$ -ketomonocarboxylic acids. If there is any significance in the individual values then  $\alpha$ -ketovaleric acid and pyruvic acid are decarboxylated only 69 and 80 % as rapidly as  $\alpha$ -ketobutyric acid.

Table I. *Decarboxylation of  $\alpha$ -keto-acids by alkaline-washed yeast*

1.0 ml. washed yeast suspension; 0.10 ml.  $\text{MgCl}_2$  (100  $\mu\text{g}$ . Mg). Total vol. made up to 2.3 ml. with phosphate buffer, pH 6.2.

Exp. 211 (1 exp. out of 3 quoted)

	CO <sub>2</sub> production in 25 min. ( $\mu\text{l}$ .)	
	Nil	Cocarboxylase (2 $\mu\text{g}$ .)
Residual	1	1
Pyruvate	19	316
$\alpha$ -Ketobutyrate	21	396
$\alpha$ -Ketovalerate	12	273

Exp. 217 (1 exp. out of 2 quoted)

CO<sub>2</sub> production ( $\mu\text{l}$ .) in 30 min.

	Nil	Vitamin $\text{B}_1$ (10 $\mu\text{g}$ .)	Cocarboxylase (1 $\mu\text{g}$ .)	Cocarboxylase (1 $\mu\text{g}$ .) and V (10 $\mu\text{g}$ .)
Residual	1	8	1	3
Pyruvate	27	31	181	465
$\alpha$ -Ketobutyrate	17	23	189	503

Exp. 212 (1 exp. out of 2 quoted)

	Nil	Vitamin $\text{B}_1$ (10 $\mu\text{g}$ .)	Cocarboxylase (1 $\mu\text{g}$ .)	Cocarboxylase (1 $\mu\text{g}$ .) and V (10 $\mu\text{g}$ .)
Residual	1	6	1	1
$\alpha$ -Ketobutyrate	22	27	165	548
$\alpha$ -Ketovalerate	17	11	122	386

#### THE INTERACTION BETWEEN $\alpha$ -KETO-ACIDS AND THE PYRUVATE DEHYDROGENASE FROM BRAIN

The question now arises as to whether the pyruvate dehydrogenase from brain will react with other  $\alpha$ -keto-acids, and if so, whether to the same degree as found for yeast. Lipmann [1937] showed that in the presence of methylene blue under anaerobic conditions, pyruvic acid was converted by the pyruvate dehydrogenase into acetic acid and  $\text{CO}_2$ . It was also found that a simple washing would produce a preparation from pigeon's brain showing a reduction time with pyruvate and methylene blue only three times more rapid than in the absence of pyruvate. This finding has recently been confirmed [Peters & Wakelin, 1938]. Under such conditions the presence of residual substrates in the tissue was objectionable. By washing with 0.2 % KCl (see Appendix with R. W. Wakelin) the reduction time of the residue was very much increased. The technique finally used was as follows.

Normal pigeon cerebrum was finely minced ice-cold, washed three times with ice-cold Ringer phosphate pH 7.3, three times with 0.3 % KCl and once with 0.2 % KCl. During each washing the tissue was allowed to remain in contact

with the salt solution for about 15 min. so that diffusion of the substrates into the latter could take place. The bulky white suspension was shrunk by a final washing with Ringer phosphate. Observations were made *in vacuo* in Thunberg tubes at 38°, 50 mg. tissue and 0.2 ml. (40 µg.) methylene blue being used. The keto-acids were tipped in from the hollow stopper after evacuation.

Table II. *Reactivity of α-keto-acids with the pyruvate dehydrogenase from pigeon's brain*

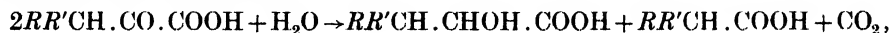
(2 exps. out of 3 quoted)

	Reduction times (min.)	
	Exp. 164	Exp. 170
Residual	167	164
α-Ketovalerate	104	60
Pyruvate	14	10½, 9½
α-Ketobutyrate	15	11½
Pyruvate + α-ketobutyrate	10	10½
Pyruvate + α-ketovalerate	—	10

The experiments in Table II show that pyruvate and α-ketobutyrate react at the same rate with the pyruvate dehydrogenase system, strongly indicating that the active centre is the same for both. α-Ketovalerate shows an almost negligible activity (15%), which is quite distinct from the slight reduction found for yeast. This inactivity cannot in any way be due to the effect of a poison, since the reduction time for pyruvate is not altered by the presence of α-ketovalerate. This difference seems to suggest that some essential part of the system, perhaps the protein component, is different from the corresponding part of the yeast system.

#### *α-Keto-acids and the Krebs' dismutation*

If the pyruvate dehydrogenase plays a part in the Krebs' dismutation [Krebs & Johnson, 1937],



it might be expected that α-ketovalerate would behave differently from pyruvate under these conditions. Weil-Malherbe [1937] showed that with slices of rat brain under anaerobic conditions, pyruvic acid was converted into the theoretical quantities of lactic acid and CO<sub>2</sub>.

Working with washed minced pigeon's brain, both in Krebs' bicarbonate and in Ringer phosphate saturated with CO<sub>2</sub>, we have compared the CO<sub>2</sub> evolution from pyruvic acid and the two α-keto-acids (Table III). Since we have washed the tissue in many of the experiments to be described later, the method of washing may be given here [cf. McGowan, 1937]. The brains (cerebrum and optic lobes) of three or four pigeons were finely minced ice-cold, transferred to a centrifuge tube and washed three times with ice-cold Ringer phosphate (3 × 30 ml.). After each centrifuging, the tissue was ground against the side of the tube with a glass rod. Finally, after being well mixed, it was transferred to the previously weighed experimental bottles in roughly equal quantities (270 mg.). Residual respiration was much reduced by this treatment, without impairing the activity of the system for metabolizing pyruvic acid.

The residual bicarbonate production (Exp. 168) is not increased by the presence of pyruvic or other α-keto-acid, so that the acid production is an index of the extent to which the Krebs' dismutation proceeds. It will be

Table III. *The Krebs' dismutation of  $\alpha$ -keto-acids by washed pigeon brain brei*

(a) In Krebs' bicarbonate (pH 7.3). 1 exp. out of 4 quoted. Exp. 168. Duration 120 min.

	CO <sub>2</sub> production, $\mu$ l./g.	
	Acid production	Bicarbonate production
Residual	56	36
Pyruvate	172	33
$\alpha$ -Ketobutyrate	166	35
$\alpha$ -Ketovalerate	102	40

 (b) In Ringer phosphate, saturated with CO<sub>2</sub>. pH 7.3. Exp. 229. Duration 210 min.

	Acid production
Residual	49
Pyruvate	198
$\alpha$ -Ketobutyrate	195
$\alpha$ -Ketovalerate	110

observed that with  $\alpha$ -ketobutyrate the dismutation takes place to exactly the same extent as with pyruvate, while the activity of  $\alpha$ -ketovalerate is only 40 % of this. The difference between this value and the 15 % activity of  $\alpha$ -ketovalerate towards the pyruvate dehydrogenase and methylene blue is very surprising. According to the accepted theory of the role of the pyruvate dehydrogenase in the Krebs' dismutation, identical values for  $\alpha$ -ketovalerate would be expected.

#### *Brain respiration in the presence of $\alpha$ -keto-acids*

A comparison of the increased O<sub>2</sub> uptakes of respiring pigeon brain brei due to pyruvic and other  $\alpha$ -keto-acids is given in Table IV (Exp. 206). Since variable results of small magnitude were obtained with unwashed tissue, the figures refer to washed brain, using samples of Na salts of  $\alpha$ -keto-acids of the highest degree of purity. Less pure samples gave somewhat smaller effects.

 Table IV.  *$\alpha$ -Keto-acids and brain respiration*

Washed normal brain. pH 7.3					
	O <sub>2</sub> uptake, $\mu$ l./g./hr.				
Exp. 206*	0-1	1-2	2-3	Increase	Av.
Residual	299	161	94		
$\alpha$ -Ketovalerate	313	180	116	14, 19, 22	18
$\alpha$ -Ketobutyrate	466	332	268	167, 171, 174	171
Pyruvate	1038	843	761	739, 682, 667	696
Exp. 223					
Residual	349	177	100		
0.02 M $\alpha$ -ketovalerate	381	235	147	31, 58, 47	45
0.06 M $\alpha$ -ketovalerate	337	200	133	-12, 23, 33	15
Exp. 226	0-1½	1½-3	3-4½		
Residual	297	131	78		
0.02 M $\alpha$ -ketovalerate	328	161	97	31, 30, 19	27
0.06 M $\alpha$ -ketovalerate	301	166	105	4, 35, 27	22

\* 1 exp. out of 2 quoted.

The most striking point arising from these figures is the preservation of a constant metabolic rate for  $\alpha$ -ketobutyric acid during a period of 3 hr. Under

these conditions the oxidation of pyruvic acid falls off very little, the  $O_2$  uptake being about 4 times that for  $\alpha$ -ketobutyric acid. As in previous cases,  $\alpha$ -ketovaleric acid seems to be rather inert in comparison. The  $O_2$  uptake is only about 11 % that of  $\alpha$ -ketobutyric acid, a figure approaching that found with the pyruvate dehydrogenase and methylene blue. Furthermore, increased concentration of  $\alpha$ -ketovalerate does not raise the respiration, a fall being observed in one case (Exp. 223).

We have attempted to discover the exact nature of the oxidation of  $\alpha$ -ketobutyric acid in brain by determining its R.Q. To this end, 6 manometers of the Dixon-Barcroft type, in duplicate, were used, containing

(1) Tissue alone.  $O_2$  uptake measured.  $CO_2$  absorbed by KOH.

(2) Tissue alone. Initial  $CO_2$  in solution measured by tipping in acid at time of zero reading.

(3) Tissue alone. The difference between  $O_2$  uptake and  $CO_2$  as acid production measured manometrically as respiration proceeded. Also  $CO_2$ , formed during respiration as bicarbonate, measured by tipping in acid at end of respiration period.

(4) Tissue and  $\alpha$ -ketobutyrate.  $O_2$  uptake measured as in 1.

(5) Tissue and  $\alpha$ -ketobutyrate. As for 2.

(6) Tissue and  $\alpha$ -ketobutyrate. As for 3.

In bottles 2, 3, 5 and 6  $CO_2$  was not absorbed, but Keilin cups containing 0.2 ml. 20 %  $H_2SO_4$  were provided.

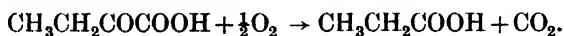
The net  $O_2$  uptake was obtained by subtracting 1 from 4. In 3 and 6 the  $CO_2$  formed by acid production was calculated from the observed reading and the corresponding  $O_2$  uptake (1 and 4). To this was added the  $CO_2$  produced in solution as bicarbonate. Initial  $CO_2$  formed in 2 and 5 was then subtracted from these totals, the difference between the final values being the net  $CO_2$  production due to oxidation of the  $\alpha$ -ketobutyrate. Experimental recordings are given in Table V.

Table V. R.Q. for brain respiration in presence of  $\alpha$ -ketobutyrate

Exp.	Duration min.	$O_2$ uptake $\mu l./g.$			$CO_2$ production $\mu l./g.$			Res. R.Q.	Net R.Q.
		Res.	$\alpha$ -Keto- butyrate	Net	Res.	$\alpha$ -Keto- butyrate	Net		
174	165	619	866	247	528	1138	610	0.85	2.46
175	180	593	877	284	505	1188	683	0.85	2.41
176	165	629	892	263	528	1138	610	0.84	2.31
179	180	548	960	412	484	1416	934	0.88	2.27
180	180	583	981	398	536	1507	971	0.92	2.44
207	180	624	1022	398	495	1510	1015	0.79	2.55
208	180	525	928	403	437	1383	946	0.83	2.36
209	220	641	1111	470	554	1636	1082	0.86	2.31
215	160	587	988	401	511	1493	982	0.87	2.45
216	160	660	1120	460	603	1648	1045	0.91	2.27
218	210	718	1212	494	675	1789	1114	0.94	2.25
Av. 180								0.87	2.37

The mean value is  $2.37 \pm 0.03$ . Agreement between individual values is not exceptionally good, but this was to be expected owing to the small differences in  $O_2$  uptake and the fact that at least 8 separate measurements have to be recorded for the calculation of a single R.Q. In addition, there seems to be a dependence of the R.Q. on the duration of the experiment. Three experiments lasting only 120 min. gave R.Q.'s as high as 2.69, and are not included.

The figures are in striking contrast to the value  $1.30 \pm 0.04$  found by McGowan [1937] for pyruvic acid, and prove undoubtedly that  $\alpha$ -ketobutyric acid is much less completely oxidized. The suggestion is that  $\alpha$ -ketobutyric acid can only be oxidized as far as the next lower fatty acid and not completely to  $\text{CO}_2$  and water as in the case of pyruvic acid:



The fact that the R.Q. found is greater than 2.0 must be due, in some measure at least, to simultaneous anaerobic dismutation producing  $\text{CO}_2$ .

A most interesting and important fact now emerges from a study of the  $\text{O}_2$  uptakes for pyruvate and  $\alpha$ -ketobutyrate under the same conditions, and which is seen in Table VI. For these two acids, the same number of  $\mu$  mol. are oxidized in a given time; in the case of  $\alpha$ -ketobutyrate, simple oxidative decarboxylation alone takes place, but in the case of pyruvate, some of the acid disappearing is completely oxidized. However, the fact is that the same rates of oxidative removal of the two keto-acids are observed, independent of the subsequent path of oxidation.

Table VI. *Comparison of the rate of oxidation of  $\alpha$ -ketobutyric and pyruvic acids*

Exp.	Duration hr.	Total $\text{O}_2$ uptake, $\mu\text{l./g.}$			$\mu$ mol. oxidized	
		Res.	$\alpha$ -Keto- butyrate	Pyruvate	$\alpha$ -Keto- butyrate	Pyruvate
206	1	299	466	1038	14.9	16.2
	2	460	798	1881	30.2	31.1
	3	554	1066	2629	45.7	45.4
213	1	307	484	1086	15.8	17.0
	2	469	831	1987	32.3	33.1
214	1	330	507	1080	15.8	16.4
	2	508	864	1989	31.8	32.4
	3	614	1149	2772	47.8	47.3
	4	686	1383	3461	62.1	60.9

The method of calculating these quantities requires some explanation. The amount of pyruvic acid disappearing by oxidative processes is given by

$$\mu \text{ mol. pyruvic acid oxidized} = \frac{x_{\text{O}_2}}{450} \times \frac{86.6}{100} \times \frac{1}{88} \times 1000 = 0.0219 x_{\text{O}_2}.$$

$x_{\text{O}_2}$  is the  $\text{O}_2$  uptake ( $\mu\text{l.}$ ); 86.6 is the % pyruvic acid disappearing by oxidative processes giving  $\text{CO}_2$  and water, and  $\text{CO}_2$  and acetic acid [Long, 1938]; the factor 450 converts  $\text{O}_2$  uptake ( $\mu\text{l.}$ ) into pyruvic acid (mg.) [McGowan, 1937], and 88 is the mol. wt. of pyruvic acid. For  $\alpha$ -ketobutyric acid the calculation is much simpler, being

$$\mu \text{ mol. } \alpha\text{-ketobutyric acid oxidized} = x_{\text{O}_2} \times \frac{2 \times 1000}{22,400} = 0.0892 x_{\text{O}_2}.$$

These results suggest very strongly that both  $\alpha$ -ketobutyric acid and pyruvic acid undergo the same initial change under aerobic conditions. It would follow from this that each gives rise to an intermediate of similar type. Whereas the intermediate from  $\alpha$ -ketobutyric acid can only break down to  $\text{CO}_2$  and presumably propionic acid, part of the pyruvic acid intermediate can be completely oxidized to  $\text{CO}_2$  and water. The probability is that this special reaction is in some way connected with the simpler structure of this intermediate, the next higher homologue of which is unable to do this owing to the hydrocarbon chain.

The high value recorded for the R.Q. of  $\alpha$ -ketobutyric acid offers a certain difficulty. This problem can now be examined more closely in the light of the

last-mentioned conclusions. For pyruvic acid, Long [1938] showed that 86.6% disappeared aerobically by oxidative reactions, and 10.4% simultaneously by the Krebs' dismutation. Since identical amounts of pyruvic acid and  $\alpha$ -ketobutyric acid disappear oxidatively, the analogy might be taken further, i.e. it seems reasonable to suppose that the extent of dismutation is also the same in the two cases, especially in view of the equal rates found under anaerobic conditions (Table III). In that case the theoretical R.Q. would be:

$$\frac{86.6 + 5.2}{0.5 (86.6)} = \frac{91.8}{43.3} = 2.12.$$

It is difficult to account for the experimental value of 2.37, but two facts may be mentioned which would lead to higher values than the theoretical:

(1) Experiments on the R.Q. of pyruvic acid gave values of c. 1.47 in cases where the tissue had not been thoroughly washed.

(2) Samples of  $\alpha$ -ketobutyric acid not of the highest degree of purity gave values of 3.59 and 3.56 (Exps. 146 and 154).

Although the effect due to 2 is probably absent from Table V, nevertheless incomplete washing of the tissue might well account for the anomaly. Lastly, judging from the influence of duration on the value of the R.Q., the calculated result might be achieved by neglecting the first  $\frac{1}{2}$  hr. of the respiration.

Some experiments have been carried out to compare the extent of Krebs' dismutation taking place under aerobic and anaerobic conditions in pyruvate solutions. The amount of dismutation proceeding aerobically was calculated from the observed  $O_2$  uptake (10.4% of the pyruvic acid disappears by dismutation). It was found that the amount of anaerobic dismutation decreased with time from about 23% during the 1st hour to 17% after 3 hr., the % referring to the anaerobic pyruvate disappearance compared with the pyruvate metabolized aerobically. Experiments have thus been continued for 3-4 hr., since this was the period in which 10.4% dismutation was found to take place aerobically [Long, 1938], Table VII; cf. also Barron & Lyman [1939].

Table VII. *Dismutation of pyruvic acid under aerobic and anaerobic conditions*

Ringer phosphate, pH 7.3; for anaerobic experiments this was saturated with  $CO_2$ .

Exp.	Duration min.	Anaerobic		Aerobic	
		Net $CO_2$ $\mu$ l./g.	Pyruvate $\mu$ mol.	Net $O_2$ $\mu$ l./g.	Pyruvate $\mu$ mol.
226	180	99	8.8	1985	5.2
227	180	81	7.2	1816	4.8
230	200	111	9.9	2183	5.7

Thus there seems to be little doubt that more dismutation of pyruvate takes place under anaerobic conditions than in the presence of  $O_2$ . It was for this reason that we could not assume identical rates of dismutation of  $\alpha$ -ketobutyric acid in the presence and absence of  $O_2$  and hence calculate the "aerobic R.Q." directly.

#### *$\alpha$ -Ketobutyric acid and the catatorulin effect with avitaminous brain*

With washed avitaminous brain, small but definite catatorulin effects were observed on adding vitamin  $B_1$  to the tissue respiring in solutions of  $\alpha$ -ketobutyrate. With  $\alpha$ -ketovalerate the effect was negligible. No catatorulin effects

were observed with the washed avitaminous brain alone. Table VIII also shows the lowered level of respiration of the avitaminous brain in the presence of  $\alpha$ -ketobutyric acid, a phenomenon so far unexplained.

Table VIII. *Catatorulin effects with  $\alpha$ -ketobutyric acid*

pH 7.3, 38°. 4 $\mu$ g. vitamin B <sub>1</sub> used						
	O <sub>2</sub> uptake, $\mu$ l./g./hr.					Catatorulin effects (last 2 hr.)
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2	2-3	
Exp. 199						
Residual	434	322	253	206	141	
Residual + vitamin	417	335	249	189	142	-4, -17, 1
$\alpha$ -Ketobutyrate	293	219	187	144	123	
Same + vitamin	312	261	222	181	164	35, 37, 41
Exp. 147	0- $\frac{1}{2}$	$\frac{1}{2}$ -1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2 $\frac{1}{2}$	2 $\frac{1}{2}$ -3 $\frac{1}{2}$		
Residual	387	232	124	69		
Residual + vitamin	400	234	124	71		2, 0, 2
$\alpha$ -Ketobutyrate	225	136	79	50		
Same + vitamin	268	172	109	71		36, 30, 21

The catatorulin effects are seen to be very small. However, mainly owing to the increased water content of the tissue due to the conditions of washing, pyruvic acid has a catatorulin effect of only 160-200  $\mu$ l./g./hr. (see Table X).

#### *Effect of $\alpha$ -keto-acids on pyruvate respiration in brain*

Working with the unwashed brain brei from avitaminous pigeons, at pH 7.3 and 38°, Peters [1937] obtained evidence that  $\alpha$ -ketobutyric acid inhibited the catatorulin effect. We have continued this work and have studied in detail the action of this acid and of  $\alpha$ -ketovaleric acid on pyruvate respiration in normal and avitaminous brain.

Figures are quoted for the normal brain in Table IX. The effects are due to pure samples of  $\alpha$ -keto-acids and are of the same order for both washed and unwashed tissue.

Table IX. *The inhibition of pyruvate respiration in brain by  $\alpha$ -keto-acids. Normal brain*

I. Unwashed tissue							
	O <sub>2</sub> uptake, $\mu$ l./g./hr.					Net pyruvate oxidation (last hr.)	Av.
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2			
(a) $\alpha$ -Ketobutyric acid. 1 exp. out of 4 quoted.							
Exp. 196							
Residual	1366	1120	883	677	436		
Pyruvate	2553	2353	2173	1977	1845	1300, 1409	1355
$\alpha$ -Ketobutyrate	1411	1272	1049	897	744		
Pyruvate + $\alpha$ -ketobutyrate	2522	2290	2128	1991	1783	1094, 1039	1067
(b) $\alpha$ -Ketovaleric acid. 1 exp. out of 5 quoted.							
Exp. 197							
Residual	1414	1178	898	719	490		
Pyruvate	2833	2580	2330	2075	1904	1356, 1414	1385
$\alpha$ -Ketovalerate	1206	1054	832	674	500		
Pyruvate + $\alpha$ -ketovalerate	2733	2475	2220	1913	1779	1239, 1279	1259



Table IX (cont.)

II. Washed tissue						
O <sub>2</sub> uptake, $\mu$ l./g./hr.					Net pyruvate oxidation (last 1½ hr.)	Av.
<div> <div>0-½</div> <div>½-1</div> <div>1-1</div> <div>1-2</div> </div>						
(a) $\alpha$ -Ketobutyrate. 1 exp. out of 3 quoted.						
Exp. 198						
Residual	483	337	276	178		
Pyruvate	1115	1026	921	761	645, 583	614
$\alpha$ -Ketobutyrate	609	522	463	371		
Pyruvate + $\alpha$ -ketobutyrate	1126	1020	920	802	457, 431	444
O <sub>2</sub> uptake, $\mu$ l./g./hr.					Net pyruvate oxidation (last 2 hr.)	Av.
<div> <div>0-½</div> <div>½-1</div> <div>1-2</div> <div>2-3</div> </div>						
(b) $\alpha$ -Ketovalerate. 1 exp. out of 2 quoted.						
Exp. 221						
Residual	421	274	186	102		
Pyruvate	1092	990	871	743	685, 641	663
$\alpha$ -Ketovalerate	424	300	207	133		
Pyruvate + $\alpha$ -ketovalerate	1089	962	801	628	594, 495	545
(c) Comparison of $\alpha$ -ketobutyrate and $\alpha$ -ketovalerate.						
Exp. 223						
Residual	416	281	177	100		
Pyruvate	1139	1027	915	792	738, 692	715
$\alpha$ -Ketovalerate	435	326	236	148		
Pyruvate + $\alpha$ -ketovalerate	1175	1011	812	607	576, 459	518
$\alpha$ -Ketobutyrate	615	476	378	311		
Pyruvate + $\alpha$ -ketobutyrate	1139	1006	850	703	472, 392	432

One conclusion which may be drawn from these figures is that  $\alpha$ -ketobutyrate inhibits pyruvate respiration more than does  $\alpha$ -ketovalerate. For  $\alpha$ -ketobutyrate the inhibition with unwashed brain is c. 21 % and with washed brain c. 38 %; in the case of  $\alpha$ -ketovalerate the corresponding figures are 9 and 23 %, at concentrations of c. 0.02 *M* in both cases. This difference would be expected from the mechanism of the inhibition (see later). It might be mentioned that in earlier experiments, in which  $\alpha$ -keto-acids of doubtful purity were used, similar net inhibitions were observed; in addition, however, the residual respiration level in the unwashed brain was lowered by  $\alpha$ -ketobutyrate, also, the extent of such inhibition decreasing with time.

Turning to the avitaminous brain, we have observed inhibitions of the catatorulin effect in pyruvate solutions by the  $\alpha$ -keto-acid homologues. The impure acids gave inhibitions of c. 30 % with unwashed brain, similar to those found by Peters [1937]. The figures quoted in Table X are for washed avitaminous brain using purest samples of the  $\alpha$ -keto-acids.

Here again the inhibition of the catatorulin effect is greater with  $\alpha$ -ketobutyrate (37 %) than with  $\alpha$ -ketovalerate (27 %). Incidentally, the real inhibition of the catatorulin effect is slightly greater than that given in Table X, owing to the fact that the catatorulin effect of the  $\alpha$ -ketobutyric acid alone has not been taken into account. The inhibiting effects produced by  $\alpha$ -ketobutyrate would then be increased by about 36  $\mu$ l./g./hr. In the case of  $\alpha$ -ketovalerate, the correction on this account would be negligible.

As to the nature of the inhibition, two alternatives seemed possible; either the  $\alpha$ -keto-acids were exerting a general effect on the respiration by inhibiting the action of the indophenol oxidase system; or the effect was peculiar to pyruvate respiration, i.e. competitive inhibition, such as has been observed in

Table X. *Inhibition of the catatorulin effect in washed avitaminous brain by  $\alpha$ -keto-acids*4  $\mu$ g. vitamin B<sub>1</sub> used. pH 7.3

	O <sub>2</sub> uptake μl./g./hr.			Catatorulin effects (last 2 hr.)	Av.
	0-1	1-2	2-3		
(a) α-Ketobutyric acid. 1 exp. out of 2 quoted.					
Pyruvate	582	405	310		
Pyruvate + vitamin	745	603	513	198, 203	201
Pyruvate + α-ketobutyrate	496	390	247		
Same + vitamin	618	520	389	130, 142	136
(b) α-Ketovaleric acid.					
Exp. 238					
Pyruvate	655	479	374		
Pyruvate + vitamin	813	677	566	198, 192	195
Pyruvate + α-ketovalerate	681	474	334		
Same + vitamin	798	622	472	148, 138	143

brain with lactate [Jowett & Quastel, 1937] and succinate [Quastel & Wheatley, 1931] in the presence of hydroxymalonic and malonic acids respectively. The effect on unwashed brain brei respiring in succinate seemed to favour the first view since an inhibition of the succinodehydrogenase system was observed comparable in magnitude with that found for the pyruvate oxidase system (Table XI).

However, when washed tissue was used, the inhibition of the succinodehydrogenase system was considerably reduced. In one exp. (186, Table XI) no inhibition was observed at all.

Table XI. *Effect of  $\alpha$ -keto-acids on succinate respiration in normal brain. S=succinate*

## I. Unwashed tissue

(a)  $\alpha$ -Ketobutyric acid. 1 exp. out of 3 quoted.

	O <sub>2</sub> uptake, $\mu$ l./g./hr.					Net succinate oxidation (last hr.)	Av.
	0-1	1-1	1-1	1-1	1-2		
Exp. 192							
Residual	1194	1010	776	599	494		
Succinate	2755	2262	1833	1290	1146	691, 652	672
$\alpha$ -Ketobutyrate	1290	1100	896	760	676		
S + $\alpha$ -ketobutyrate	2538	2110	1776	1373	1067	613, 391	502

(b)  $\alpha$ -Ketovaleric acid. 1 out of 3 quoted.

	O <sub>2</sub> uptake, $\mu$ l./g./hr.					Net succinate oxidation (last hr.)	Av.
	0-1	1-1	1-1	1-1	1-2		
Exp. 195							
Residual	1430	1059	867	627	490		
Succinate	2872	2354	1833	1380	1117	753, 627	690
$\alpha$ -Ketovalerate	1127	956	780	600	483		
S + $\alpha$ -ketovalerate	2188	1990	1520	1137	957	537, 474	506

Table XI (*cont.*)

## II. Washed tissue

(a)  $\alpha$ -Ketobutyric acid.

	O <sub>2</sub> uptake, $\mu$ l./g./hr.				Net succinate oxidation (last hr.)	Av.
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-1 $\frac{1}{2}$	1 $\frac{1}{2}$ -2		
Exp. 186						
Residual	384	266	190	—		
Succinate	782	607	489	—	341, 299	320
$\alpha$ -Ketobutyrate	592	457	382	—		
S + $\alpha$ -ketobutyrate	936	798	692	—	341, 310	325
Exp. 239						
Residual	460	293	206	172		
Succinate	880	718	587	490	381, 318	350
$\alpha$ -Ketobutyrate	611	466	383	338		
S + $\alpha$ -ketobutyrate	1041	825	689	566	306, 228	267

(b)  $\alpha$ -Ketovaleric acid. 1 exp. out of 2 quoted.

	O <sub>2</sub> uptake, $\mu$ l./g./hr.				Net succinate oxidation (last 2 hr.)	Av.
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	1-2	2-3		
Residual	409	279	181	102		
Succinate	784	650	506	348	325, 246	286
$\alpha$ -Ketovalerate	452	354	237	148		
S + $\alpha$ -ketovalerate	789	657	469	321	232, 173	203

The absence of any general effect on the indophenol oxidase system was conclusively proved by our finding that  $\alpha$ -ketobutyric acid had no action on the respiration of pig's heart muscle extract in the presence of cytochrome *c* and quinol (Table XII).

Table XII.  $\alpha$ -Ketobutyric acid and the indophenol oxidase

Exp. 183. 1 exp. out of 2 quoted.

Quinol mg.	Indophenol oxidase ml.	Cytochrome <i>c</i> ml.	$\alpha$ -Keto- butyrate mg.	$\mu$ l./g./hr.	
				0- $\frac{1}{2}$	$\frac{1}{2}$ - $\frac{1}{2}$
5	—	—	—	101	76
5	—	—	5	73	85
5	0.20	—	—	632	327
5	0.20	—	5	656	338
5	0.20	0.10	—	782	177
5	0.20	0.10	5	784	153

Finally, it was observed that the inhibition of pyruvate respiration in washed normal brain was increased on tripling the concentration of the  $\alpha$ -ketobutyric acid (Table XIII).

Table XIII. *Effect of increasing the concentration of  $\alpha$ -ketobutyric acid on pyruvate respiration in washed normal brain*

	O <sub>2</sub> uptake, $\mu$ l./g./hr.				Net pyruvate oxidation (last 1 $\frac{1}{2}$ hr.)	Av.
	0- $\frac{1}{2}$	$\frac{1}{2}$ -1	$\frac{1}{2}$ -1	1-2		
Exp. 198						
Residual	483	337	276	178		
Pyruvate	1115	1026	921	761	645, 583	614
0.02 <i>M</i> $\alpha$ -ketobutyrate	609	522	463	371		
Same + pyruvate	1126	1020	920	802	457, 431	444
0.06 <i>M</i> $\alpha$ -ketobutyrate	602	526	475	371		
Same + pyruvate	1037	963	855	680	380, 309	345

On increasing the concentration of  $\alpha$ -ketobutyric acid from 0.02 to 0.06 *M*, the inhibition of pyruvate respiration is raised from 28 to 44 %.

Although there has been no attempt to work out the kinetics in detail, there can be little doubt that competitive inhibition is taking place. It has already been shown that the initial stage in  $\alpha$ -keto-acid oxidation is the same for pyruvic acid and  $\alpha$ -ketobutyric acid. If the two are allowed to compete for an enzyme, they will do so in proportion to their individual concentrations. In brain tissue under aerobic conditions, the amounts of the corresponding intermediates formed will be in this same ratio. Since the breakdown of the  $\alpha$ -ketobutyric acid intermediate is unaccompanied by an uptake of  $O_2$ , an inhibition of pyruvate respiration will be observed the magnitude of which will depend on the relative concentrations.

In the avitaminous brain, inhibition is also observed, and this can only mean that both  $\alpha$ -ketobutyrate and pyruvate are competing for the centres activated by vitamin  $B_1$ . Taken separately they both show catatorulin effects, that due to  $\alpha$ -ketobutyrate being smaller than in the case of pyruvate. We are thus led to the conclusion that vitamin  $B_1$  is essential for the initial stage in the oxidation. Support for this proposed mechanism of the catatorulin effect is to be found in the fact that the % inhibition of pyruvate respiration in normal brain and the catatorulin effect with pyruvate in the avitaminous brain by  $\alpha$ -ketobutyrate are approximately the same (c. 35 %).

The case of  $\alpha$ -ketovalerate can be provisionally explained. This substance is oxidized to a small extent only by the normal and avitaminous brain, which suggests that its rate of forming the corresponding intermediate is low. Thus one would expect the competition with pyruvate to be smaller, and this is what has been found. Such a view, however, is not quite consistent with the finding that 0.02 *M*  $\alpha$ -ketovalerate saturates the system (Table IV), an excess not increasing the rate of formation of the intermediate. Owing to the small increase in  $O_2$  uptake observed, it has not been possible to determine the saturation concentration of  $\alpha$ -ketovaleric acid in the brain system, but by analogy this is probably considerably below 0.02 *M*.

### DISCUSSION

In order to clarify the various points concerning the effect of  $\alpha$ -ketobutyric and  $\alpha$ -ketovaleric acids on the respiration of pyruvate and succinate in washed and unwashed brain, the following Table XIV is appended. Effects due to samples not of the highest degree of purity are listed in brackets whenever their behaviour differs from that of the purest specimens.

Table XIV. *Net effects of  $\alpha$ -keto-acids on the respiration of pigeon brain brei*

	Normal brain						Avitaminous brain					
	Residual		Succinate		Pyruvate		Cat. effect with pyr.		Residual			
	Unw.	Wash.	Unw.	Wash.	Unw.	Wash.	Unw.	Wash.	Unw.	Wash.	Unw.	Wash.
$\alpha$ -Ketobutyrate	+	(-)	+	-	-	-	-	-	-	-	-	-
$\alpha$ -Ketovalerate	-	-	+	0?	-	-	-	-	-	-	-	-

+ indicates increased respiration; - indicates an inhibition.

The most significant points arising out of the work described are (a) that the rate of decarboxylation by the yeast carboxylase system is practically the same for three  $\alpha$ -keto-acids, and (b) that the rate of oxidation by brain tissue is the same for two of them, pyruvic acid and  $\alpha$ -ketobutyric acid; (c) the difference

between these two lies in the further metabolism of pyruvic acid, so that part of the initial oxidation product is completely oxidized to  $\text{CO}_2$  and water. Since cocarboxylase is essential for decarboxylation by yeast, the inference from (a) and (b) is that it is also responsible for the initial change in brain. In the avitaminous brain this change is accelerated by the addition of vitamin  $\text{B}_1$ ; and in view of the recent finding by Ochoa & Peters [1938, 2] that a limited though definite synthesis of cocarboxylase from vitamin  $\text{B}_1$  takes place in surviving brain tissue, this suggestion is at least plausible. Further support for cocarboxylase as an essential constituent of the pyruvate oxidase system is to be found in the statements of Ochoa & Peters [1938, 1] and Westenbrink & Goudsmit [1938] that normal brain tissue contains *c.*  $4 \mu\text{g./g.}$  cocarboxylase, while the free vitamin  $\text{B}_1$  content seems to be negligible. The main difference between the yeast and brain systems is that in the latter case oxidation accompanies decarboxylation [cf. Lipmann, 1937]. Lastly the oxidative decarboxylation is specific,  $\alpha$ -ketovalerate being hardly affected by the pyruvate dehydrogenase in brain. From this fact, the inference must be drawn that the protein part of the enzyme is different in yeast and brain.

In regard to the methylene blue experiments, it is to be noted that pyruvic acid and  $\alpha$ -ketobutyric acid cause decoloration at practically the same rate; hence this does not follow the total  $\text{O}_2$  uptake, but only that produced by the postulated system A. Thus, support is obtained for the view that during the intensive washing with hypotonic salt solutions, the components of system B have been lost.

#### SUMMARY

1. Cocarboxylase is essential for the decarboxylation by yeast of  $\alpha$ -ketovaleric acid as well as for pyruvic and  $\alpha$ -ketobutyric acids. Vitamin  $\text{B}_1$  increases the rate of decarboxylation of  $\alpha$ -ketobutyric and  $\alpha$ -ketovaleric acids only in the presence of cocarboxylase.  $\text{CO}_2$  evolution with  $\alpha$ -ketovaleric acid is slightly less than with the others.
2. Pyruvic and  $\alpha$ -ketobutyric acids are equally reactive with the pyruvate dehydrogenase system in brain under anaerobic conditions in the presence of methylene blue.  $\alpha$ -Ketovaleric acid is much less reactive.
3. The Krebs' dismutation proceeds to the same extent with  $\alpha$ -ketobutyric acid and pyruvic acid; again  $\alpha$ -ketovaleric acid is less affected.
4. Washed brain tissue causes oxidative decarboxylation of  $\alpha$ -ketobutyric acid giving presumably propionic acid (next lower fatty acid).  $\alpha$ -Ketovaleric acid is oxidized to only a very slight extent. The purity of the acids used was of the greatest importance, high values for the R.Q. of  $\alpha$ -ketobutyric acid being obtained in the presence of a slight impurity.
5.  $\alpha$ -Ketobutyric acid and  $\alpha$ -ketovaleric acids enter into competitive inhibition with pyruvic acid both in the normal and avitaminous brains respiring *in vitro*. There is no inhibition of the indophenol oxidase system.
6. Pyruvic and  $\alpha$ -ketobutyric acids are oxidized at exactly the same rate under identical conditions. In the former case, however, most of the initial oxidation product undergoes complete combustion to  $\text{CO}_2$  and water. This indicates that there is an initial common path (A) for oxidative decarboxylation; in the case of pyruvic acid a further system (B) causes complete oxidation of part of the intermediate.

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## APPENDIX

WITH R. W. WAKELIN

Lipmann [1937] showed that thoroughly extracted rat brain catalysed the reduction of methylene blue in the presence of pyruvate; the control without pyruvate showed practically no residual reduction. Pigeon brain, on the other hand, would only yield preparations which gave about a three-fold quicker reduction time with pyruvate than the control. We have found that if the pigeon's brain tissue is washed first with Ringer phosphate, pH 7.3, and then with either 0.2 or 0.3 % KCl, a hypotonic solution, residual substrates are almost completely removed. After this washing, it is best to shrink the preparation in Ringer phosphate solution, as the hypotonic solutions lead to marked swelling of the tissue. For exact details of this preparation, see the text, p. 761. Variations of this procedure such as preliminary extraction with distilled water do not give satisfactory results. Not only substrates, but also the capacity for giving appreciable O<sub>2</sub> uptakes with pyruvate, are removed by our treatment; however, all the necessary components to give aerobic oxidation of succinic acid are present, though much reduced as compared with unwashed tissue. The pyruvate dehydrogenase present, unlike the complete oxidase system, is moderately resistant to freezing. Since O<sub>2</sub> uptake is here abolished without loss of the capacity for dehydrogenation, it is system B which is eliminated. It is interesting to note that we found in some experiments an inhibition of O<sub>2</sub> uptake with pyruvate due to methylene blue, in amounts which would give excellent results in the anaerobic experiments.

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# XCVIII. STUDIES ON THE METABOLISM OF PYRUVIC ACID IN NORMAL AND VITAMIN B<sub>1</sub>- DEFICIENT STATES

## II. BLOOD PYRUVATE LEVELS IN THE RAT, PIGEON, RABBIT AND MAN

## III. THE RELATION OF BLOOD PYRUVATE TO CARDIAC CHANGES

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## II. BLOOD PYRUVATE LEVELS IN THE RAT, PIGEON, RABBIT AND MAN

THE physiology and biochemistry of vitamin B<sub>1</sub> have been recently reviewed [Peters, 1938; Cowgill, 1938]. While the role of vitamin B<sub>1</sub> in the mammalian organism remains undefined there is now much evidence in support of the view that the primary biochemical lesion in B<sub>1</sub>-deficiency is the inability of the cell to metabolize pyruvic acid [Peters, 1936].

The accumulation of pyruvic acid in the blood in vitamin B<sub>1</sub>-deficiency was first shown by Thompson & Johnson [1935] in England, using rats and pigeons, and by Platt & Lu [1935] in China on human beri-beri cases. The above-mentioned authors were able to relate the increase of bisulphite-binding substances (B.B.S.), expressed as pyruvic acid, to B<sub>1</sub>-deficiency, by demonstrating that the raised level returns to normal after administration of vitamin B<sub>1</sub>. Pyruvic acid was isolated from the blood, cerebrospinal fluid and urine of fulminating beri-beri cases [Platt & Lu, 1935] and from the blood of avitaminous pigeons [Johnson, 1936]. Later, in a more detailed paper, Platt & Lu [1936, 1] showed that contrary to the results obtained in avitaminous animals where the large increase of B.B.S. was probably due entirely to pyruvic acid, only a fraction could be accounted for as pyruvic acid in human cases when the latter is estimated by the hydrazone method [Peters & Thompson, 1934].

In view of the evidence of the peculiar relationship of pyruvic acid to vitamin B<sub>1</sub>, Platt & Lu [1936, 1] suggested that further investigations, especially of pyruvic acid itself, were needed before raised B.B.S. values could be reasonably attributed to lack of vitamin B<sub>1</sub>. The later investigations of Platt & Lu [1936, 2] on the accumulation of pyruvic acid and the effect of vitamin B<sub>1</sub> on the pyruvic acid and carbonyl compounds in beri-beri blood clearly showed that the initial resting level of blood pyruvic acid gave an indication of the degree of deficiency. Furthermore the B.B.S. value did not so readily return to its normal level after intravenous B<sub>1</sub>-injection as compared with pyruvate. In this connexion, it may be mentioned that de Jong [1936] found that the disappearance of symptoms

after the administration of vitamin B<sub>1</sub> to his pigeons sometimes preceded the lowering of bisulphite-binding capacity.

Since then Wilson & Ghosh [1937] have published evidences of increased blood B.B.S. in cases of epidemic dropsy, but the contribution of pyruvic acid to this increase is as yet unknown. Wilkins *et al.* [1937], who found an elevation of B.B.S. in conditions associated with ketosis, anoxaemia and uraemia, as well as B<sub>1</sub>-deficiency, also concluded that the estimation of blood B.B.S. is not specific for B<sub>1</sub>-deficiency.

Owing to the complicated procedure involved in the quantitative estimation of pyruvic acid by the Neuberg-Case hydrazone method, and in spite of the accumulating evidences of the possible role of vitamin B<sub>1</sub> as its pyrophosphate in the decarboxylation and dehydrogenation of pyruvic acid in the mammalian organism, there exists in the literature very little information with regard to the actual change of pyruvate in the blood or tissues of the B<sub>1</sub>-deficient animals. This quantitative conception is of importance for a proper appreciation of the role of pyruvate in the metabolism of the normal and B<sub>1</sub>-deficient individuals.

### *Experimental details*

Albino rats, weighing about 70–90 g., were devitaminized with a diet of the following composition: casein 18 %, salt 4 %, agar 2 %, cod liver oil 2 %, arachis oil 3 %, sucrose 71 %, and addition of 60 ml. of autoclaved marmite 100 g. (Marmite solution: mix 5 g. marmite with 10 ml. 43.5 % NaOH and 90 ml. water, autoclave for 1 hr.) Electrocardiograms were taken according to the method of Birch & Harris [1934]. Blood samples were obtained by decapitating the rats and collecting the blood in ice-cold trichloroacetic acid before the estimation. Pigeons were fed on thrice washed polished rice and marmite (3 g. per day) plus 1 drop of radiostoleum. Blood samples were obtained by decapitation after marked head retraction had developed. Rabbit blood was obtained from the ear vein. In view of the effect of muscular activity on blood pyruvate (see Tables III, IV) special care was taken to keep the animals under resting conditions in a dark room for at least 1 hr. before the collection of the blood. The rabbit was therefore kept during this time in a box which was just the size of its body so as to eliminate the possibility of its walking about in the cage. With the exception of a few acute beri-beri cases all specimens of blood from the human subjects were obtained after the patient had more than  $\frac{1}{2}$  hr. of rest in bed.

All pyruvate estimations were made by the specific hydrazone method previously described [Platt & Lu, 1936, 1; Lu, 1939].

Pyruvate determinations in the blood of various species of normal and avitaminous states are summarized in Tables I and II.

Table I. *Pyruvate in blood of normal and avitaminous animals*

	mg. pyruvic acid per 100 g. blood	
	Normal	Avitaminous
Pigeons	0.87 (5)	5.39 (5)
Rats	1.09 (5)	3.21 (4)
Rabbit	0.98 (11)	—

The numbers in brackets denote the number of estimations. Subcutaneous injection of 100 i.u. of vitamin B<sub>1</sub> to rats restores the blood pyruvate level to normal in 4–6 hr.; 250 i.u. to pigeons only after 24 hr.



Table II.\* *Pyruvate in blood of healthy and vitamin B<sub>1</sub>-deficient human subjects*

State of deficiency	mg. pyruvic acid per 100 g. blood			
	No. of cases studied	Range	Mean	Standard deviation
Cured (normal)	60	0.4-0.75	0.55	±0.12
Subacute (chronic)	84	0.77-1.93	0.93	±0.40
Fulminating (acute)	38	1.00-5.77	2.72	±1.13

\* The author is much indebted to Dr B. S. Platt in collaboration with whom the data in Table II were obtained.

All the cases studied had at one time shown some degree of deficiency. For the assessment of symptoms in terms of vitamin B<sub>1</sub>-deficiency and the relation of the value of this test to clinical studies see Platt & Lu [1936, 1]. Several hours (6-8) are required to initiate the removal of pyruvic acid after intravenous injection of 1000 or 2000 I.U. of vitamin B<sub>1</sub>.

#### *Effect of exercise on blood pyruvate*

The effect of exercise on blood pyruvate is interesting and important. Food intake and muscular activity are known to be associated with the increase in carbohydrate metabolism which leads to increased requirement for vitamin B<sub>1</sub>. In experiments with beri-beri cases, muscular activity appears to be one of the many factors which contribute to the development of acute symptoms. Strenuous exercise in man also leads to increase of pyruvic acid in the blood and an increased heart rate.

Table III. *Blood pyruvate of rabbits under basal and normal conditions*

Rabbit no.	Body weight in kg.	Blood pyruvic acid, mg. per 100 g.		
		Basal	Normal	Difference
1	1.257	1.03	1.43	0.40
2	1.437	1.36	1.89	0.53
3	1.321	0.90	1.48	0.58
Average	1.434	1.09	1.60	0.51

From Table III it can be seen that the basal level (fasting, immobile) rises by 50 % when the animal is allowed to feed and move about. Table IV shows that the average increase of pyruvate in the blood after 3 min. exercise is 4.68 mg. %. It required the injection of 180 mg. of pyruvic acid to produce the same rise. We must conclude, therefore, that at least 180 mg. are liberated during the 3 min. exercise. A normal rabbit can remove this in 30 min. This is in agreement

Table IV. *Blood pyruvate of rabbits under normal conditions and after 3 min. exercise*

Rabbit no.	Blood pyruvic acid, mg. per 100 g.		
	Normal	After 3 min. exercise	Increase
1	1.46	4.98	3.52
2	1.33	2.74	3.41
3	1.23	4.32	3.09
Average	1.34	4.68	3.34

N.B. The exercise consisted of holding the rabbit by the ear and letting it kick: when it refuses to do so it is brought on to a round glass jar where it struggles to find steady footing.

with the results of Johnson & Edwards [1937] on the exercise effect in healthy men, and of Wilkins *et al.* [1937] on pyruvate injected into normal men. Excess pyruvate in the blood up to 100 mg. per kg. body weight can be removed from the blood stream in 30 min. This must mean that 6 g. can be formed in a normal man in 30 min.

### DISCUSSION

Pyruvic acid appears in very small quantities under resting normal conditions. Maintained raised levels are found in various states of hypovitaminosis and a temporary raised level after muscular activity. Administration of vitamin B<sub>1</sub> restores the raised blood pyruvate levels to normal as the apparent acute symptoms disappear. Wilkins *et al.* [1938] studied three cases with nutritional deficiency which gave pyruvic acid values by the hydrazone method of 0.73, 0.46 and 0.72 mg. per 100 ml. It will be seen from Table II that these values are in the order of the non B<sub>1</sub>-deficiency range in man. So far as is known at the present time pyruvic acid increase in the blood under *resting* conditions has not yet been detected in any pathological state other than B<sub>1</sub>-deficiency. We therefore appear to be justified in assuming that pyruvic acid accumulation in the blood is specific for B<sub>1</sub>-deficiency. The test has been used in clinical studies of vitamin B<sub>1</sub>-deficiency in man [Platt, 1938].

It will be seen from Table II that a maintained raised level of blood pyruvate is associated with vitamin B<sub>1</sub>-deficiency in man. The fact that a very small amount of difference is obtained between the normal and the subacute cases and the complicated procedure for estimation of pyruvic acid by the hydrazone method have so far limited the applicability of this finding for assessing the state of B<sub>1</sub>-deficiency in clinical studies. However, the latter objection has now been met by the introduction of the new micro-estimation method for pyruvic acid in blood [Lu, 1939] and the former can be overcome by an exercise tolerance test. Platt & Lu [1936, 2] observed an increase of blood pyruvic acid following exercise in both normal and deficient human subjects; the level in the normal rapidly returns to its usual value, while in the deficient subject increased values are maintained or even further raised for some time after. The details of an exercise tolerance test based on this will be reported in the next paper of this series.

However, it must be remembered that there is evidence of increase of pyruvic acid in the blood and urine of healthy individuals after severe exercise [Johnson & Edwards, 1937; Lu & Platt, 1936], and in the urine of uncompensated diabetic patients [Pi Suñer & Farran, 1936]. Furthermore, no significant change of blood pyruvate could be detected in the "atrophic type" of beri-beri where the nerves are primarily involved [Platt & Lu, 1936, 1] and which is supposed to be also due to vitamin B<sub>1</sub>-deficiency, although the natural history of this type is not yet clear.

This requires some comment. Careful consideration of the data given in Table II shows that blood pyruvate values of some of the subacute cases come well within the range of values assigned for the fulminating cases. A detailed clinical discussion of some of these has been published [Platt & Lu, 1936, 1], others will be discussed in a separate report. For the present purpose it will suffice to mention that careful analyses of the biochemical and symptomatic syndromes of a large number of beri-beri cases indicate that this lack of identical behaviour in various types of deficiency may well be due to such factors as fever, toxæmia, endocrine disturbances and inanition, which are known to entail abnormal carbohydrate metabolism. Other vitamin deficiencies, e.g. A and C, were in some instances identified as existing in the same patient, especially

among the chronic, subacute type. How far other factors affect the whole syndrome is not known. Thus before it can be concluded whether increased pyruvate in the blood is specific for  $B_1$ -deficiency it is necessary to single out characteristic biochemical and symptomatic syndromes and to correlate them as specific conditions for the identification of the deficiency in an organism where other pathological conditions are likely to play an important part. For example, it might be said that the presence of pyruvic acid in diabetic urine gives ground for doubting the validity of using pyruvic acid estimations for research into vitamin  $B_1$ -deficiency states. But on the other hand the increase in urinary pyruvate may well be due to secondary causes; thus the altered water metabolism may affect the individual in such a way that his daily requirement of vitamin  $B_1$  is increased: hence his ordinary intake which may be sufficient for a normal individual becomes insufficient, and a state of unrecognized induced or secondary avitaminosis is set up. Of course there is also the possibility of a primary disturbance of the intermediate carbohydrate breakdown which would also involve the accumulation of pyruvic acid.

Studies of the permutations and combinations of the numerous factors which may accompany vitamin  $B_1$ -deficiency or be involved in it are urgently needed. In the meantime, inferences drawn from the study of certain biochemical and clinical syndromes are useful as an aid to further advance, though they can only be regarded as preliminary and must be interpreted with caution. Nevertheless estimation of pyruvic acid in the blood forms a practical method for assessing the state of  $B_1$ -deficiency in man.

In the following section an examination is made of the blood pyruvate in correlation with the functional manifestation of cardiac disturbance under various conditions of  $B_1$ -deficiency with and without inanition or toxæmia and also under normal conditions. In the latter, the increase of blood pyruvic acid was produced from without by injections of Na pyruvate. The results account to some extent for some of the obscure combinations of conditions mentioned above.

### III. THE RELATION OF BLOOD PYRUVATE TO CARDIAC CHANGES

In the case of vitamin  $B_1$ -deficiency, one of the most important and prominent symptoms is the disturbed function of the heart. The disturbances are not the same in all species; thus in pigeons (fed on polished rice) a vagal heart block seems to occur [Carter & Drury, 1929]; or bradycardia [Sankaran & Krishnan, 1936]; and in rats bradycardia of sinus origin [Drury *et al.* 1930]. While the cardiac changes in pigeons have been attributed to a special pigeon factor the bradycardia in rats is generally held to be specific for  $B_1$ -deficiency. Cardiac change in human beri-beri had long been known and was described in detail [Asslmeier & Wenckebach, 1928; Wenckebach, 1934; and Hashimoto, 1937]. Enlargement of the right side of the heart regularly occurs in human cases but has been observed only once in pigeons [McCarrison *et al.* 1928]. Studies on the nature of the electrocardiographic changes in rats [Zoll & Weiss, 1937] and in man [Raman, 1936; Weiss *et al.* 1936; Weiss & Wilkins, 1937] have shown that the cardiac changes in rats are similar to those observed in man. The essential difference is that in man a tachycardia exists. Platt & Lu [1936, 1, 2] reported

that this tachycardia of severe beri-beri cases gave place to a transient bradycardia during recovery after treatment with vitamin B<sub>1</sub>. Weiss & Wilkins [1937] have made a systematic study of the nature of the various cardiovascular disturbances and the effect of vitamin B<sub>1</sub> on them. Cardiac changes existing in cases with no B<sub>1</sub>-deficiency symptoms did not respond to B<sub>1</sub>-administration. They state that, as far as is known, B<sub>1</sub>-hypovitaminosis is the only vitamin deficiency which is followed by cardiac disturbances. They think it probable that the myocardial disturbances reported in rickets and scurvy are caused by simultaneous vitamin B<sub>1</sub>-deficiency. However, there is also evidence of a tachycardia attributable to vitamin C-deficiency in guinea-pigs [Sankaran & Krishnan, 1936] and a bradycardia in rats attributable to inanition and not curable by vitamin B<sub>1</sub> [Parade, 1937].

Cowgill, on the other hand, has evidence that the heart is not involved in B<sub>1</sub>-deficiency in dogs. While it is important to recognize that because tachycardia is attributable to vitamin C-deficiency in guinea-pigs it does not necessarily follow that vitamin C-deficiency would give rise to tachycardia in man or other mammals; yet it is clear that unless coexisting pathological conditions such as other deficiencies are known, the specific biochemical lesion due to the deficiency of one entity cannot be solved. In animal experiments this difficulty is not so serious, although it arises on account of our still relatively imperfect knowledge of synthetic diets. When dealing with human cases, the probability of mixed deficiency is much greater. Knowledge of factors modifying typical biochemical or functional changes is of great importance in recognizing the conditions.

The restoration of cardiac function in human B<sub>1</sub>-deficiency can vary a great deal; while the apparent acute symptoms disappear as the pyruvate level in the blood returns to normal, yet cardiac function may or may not be normal even after several days. It was observed in a few of the chronic beri-beri cases where the heart did not immediately resume its normal function after vitamin B<sub>1</sub> treatment, it slowly did so after a period of large vitamin C intake (given for other experiments). Some patients, however, also recovered after various times without this addition of vitamin C. Whether this delayed cure is due to the accompanied dietary change after B<sub>1</sub> or organic changes in the heart tissue itself resulting from chronic deficiency is not yet known.

In an attempt to correlate the biochemical lesion and the clinical manifestation of B<sub>1</sub>-deficiency Lu & Dju [1937] studied the relation of blood pyruvate level and the heart rate in rats, and reported that the lowered pulse rate is in most cases related to the accumulation of pyruvate in the blood of the B<sub>1</sub>-deficient rats.

In the present study, blood pyruvic acid values of rats kept under different conditions were determined, and are given in the following tables.

Table V. *Pyruvate in the blood of acute deficiency rats*

No. of rats examined	Pulse rate per min.	mg. pyruvic acid per 100 g.	
		Range	Average
5	550-500	0.98-1.19	1.09 (normal)
1	500-450	1.11	1.11
3	450-400	0.99-1.65	1.25
3	400-350	1.58-1.61	1.60
3	350-300	1.82-2.69	2.20
4	300-350	2.59-4.05	3.21

On the diet given above, rats developed the deficiency in 3 weeks. In order to ascertain that the cardiac changes were due to the deficiency, vitamin B<sub>1</sub>

was subsequently given in every case to ensure a restoration to normal. All the rats were killed within 10 days from showing the first signs of cardiac change, so that no appreciable loss of body weight occurred during the experiment. This class of animals was regarded as comparable with the fulminating type of beri-beri in man.

The results given in Table V show a clear relation between the content of pyruvate in the blood and the pulse rate; the blood pyruvate increases as the bradycardia of avitaminosis B<sub>1</sub> sets in. From the normal level of about 1 mg./100 g. it rises to 4 mg./100 g. in the most extreme cases.

Table VI. *Pyruvate in blood of chronic deficiency rats*

No. of rats examined	Pulse rate per min.	mg. pyruvic acid per 100 g.	
		Range	Average
1	450-400	2.4	2.4
4	400-350	1.25-4.06	2.75
3	350-300	1.98-3.31	2.65
0	300-250	—	—
1	250-200	5.00	5.00

Table VII. *Pyruvate in blood of infected and hypovitaminous rats*

No. of rats examined	Pulse rate per min.	mg. pyruvic acid per 100 g.	
		Range	Average
1	450-400	1.82	1.82
2	400-350	0.64-3.54	1.91
2	350-300	2.00	2.00
1	250-200	2.00	2.00
3	200-150	1.7-2.38	1.79
1*	380-175†	0.82	0.82

\* Moribund rat.

† Taken 40 min. after 0.2 ml. Betaxin.

The effect of long-continued deficiency was next studied in a group of rats maintained for 2 months on the deficient diet, but allowed from time to time a supply of vitamin B<sub>1</sub> to keep them at a pulse-rate level of approximately 400 beats per min. Such rats developed marked polyneuritis, lost about a third of their body weight and were regarded as more comparable with subacute cases of human beri-beri. As Table VI shows, the blood pyruvate, in spite of a rather wide individual variation, varies with the pulse rate though the ratio is not quite the same as in the previous experiment; for a given pulse rate the pyruvate now tends to be higher. This demonstrates that a secondary condition, such as inanition, can have an effect on both the heart and the pyruvate.

Since concurrent fever and infection is not uncommon in human beri-beri, a third group of rats was studied. After a month on the deficient diet, rats become particularly susceptible to cold, and are liable to develop infections. Post mortems on such rats showed infective lesions of liver and lung. Thus the third group had avitaminosis, inanition and toxæmia.

The figures given in Table VII show that now there is little correlation to be seen between blood pyruvate and heart rate. The normal value for pyruvate may even be obtained in moribund rats which have received a dose of vitamin B<sub>1</sub> but which are continuing to show a fall of pulse rate. These observations help to account for some obscure beri-beri cases where, after prolonged illness and infection, even excessive doses of B<sub>1</sub> will not bring about effective cure.

The averages of the foregoing tables are plotted in Fig. 1, which illustrates strikingly what has been said above.

By the use of the newly developed micro-method [Lu, 1939] for the determination of pyruvic acid in small quantities of blood it was possible to follow the blood pyruvate changes in individual rats at various stages of deficiency as indicated

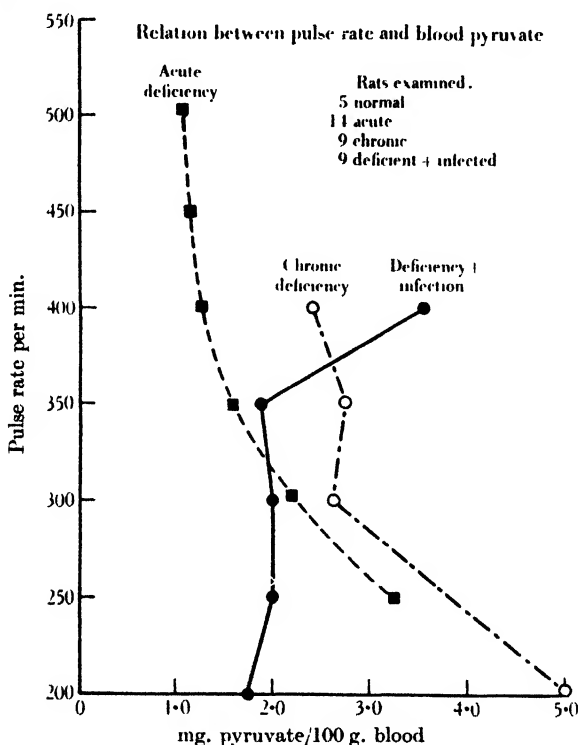


Fig. 1.

by the bradycardia, and at the different stages of recovery after the subcutaneous injection of vitamin B<sub>1</sub>. To obtain successive blood samples the following technique was found to be very serviceable: xylol was applied to the rat's tail, causing the blood vessels to dilate; after a minute or two the tail was cleaned and dried under sterile conditions. If 2 mm. of the tail tip from which 1 in. had previously been severed, are suddenly cut off, two or three drops of blood are obtained. The wound is then closed with collodion solution, and the process is repeated when more blood is required. The results from two rats rendered deficient without much loss of weight or any other complication are given in Table VIII. Similar analyses made on chronic and infected rats gave results of the same order as those described above.

Experiments of the type reported in Table VIII clearly confirm the previous experiments and show that the bradycardia of vitamin B<sub>1</sub>-deficiency in rats runs parallel with the biochemical changes observed in the blood, and that vitamin B<sub>1</sub> removes the bradycardia and restores the blood pyruvate level to normal only in pure B<sub>1</sub>-deficiency, and not in the chronic or infected rats. Birch & Harris [1934] observed the same correlation of bradycardia with lactic acid in the blood.

Table VIII

Pulse rate per min. approx.	Blood pyruvic acid, mg. per 100 g.	
	Rat I	Rat II
500-550	0.98	1.03
450-500	1.17	1.21
400-450	1.54	1.63
350-400	2.07	2.14
300-350	3.24	3.28
*350-400	2.14	2.20
*400-450	1.50	1.59
*450-500	1.21	1.30
*500-550	1.02	0.99

\* After administration of vitamin B (100 i.u. injected subcutaneously).

It is worthy of note that the return of the heart rate to normal after injection of  $B_1$  takes place within 4 hr., which seems to indicate that this cardiac disturbance is the result of a "biochemical lesion". Whether the lack of similar response of cardiac disfunction to  $B_1$ -therapy in the chronic and infected cases is attributable to organic change of the heart tissue itself is not known. In this connexion, mention may be made of the delayed return to normal function of the heart in chronic beri-beri cases.

#### *Effect of injected pyruvate on heart rate*

Next it was thought necessary to know the effect of injected pyruvate on the heart-rate. It appears from injections of pyruvate in man [Wilkins *et al.* 1937] and dogs [Flock *et al.* 1938] that no toxic effects follow, but such effects have been said to occur in the rabbit [Kermack *et al.* 1927]; this was not confirmed in the present work. Toxic effects might perhaps be expected since we know that pyruvic acid polymerides are toxic [Peters, 1936; Lipschitz *et al.* 1938], especially as the heart is a sensitive organ and readily affected by changes in the internal medium. The effect on the heart would however be likely to be transient since the metabolic disappearance of pyruvic acid from the blood stream is so rapid. The pyruvate solution used was prepared in the same manner as described previously [Lu, 1939] and injected as 40 mg./ml. in 0.9% NaCl.

Rabbits were suitable for these experiments because of the possibility of injection into the vein of one ear and the taking of the blood from the other after the shortest interval of time. Electrocardiograph tracings were taken before, during and after injection of pyruvate in amounts of 80 mg. per kg. body-weight. Of the six rabbits studied, with the exception of one which had a skin lesion at the corner of its mouth, and was later proved to be diseased, all showed no change in heart rate, even after the blood pyruvate was raised to 4 or 8 times its normal level. (See Fig. 2.) Thus it seems to follow that pyruvic acid up to 10 mg./100 g. in the blood is not toxic, and is not the cause of the cardiac disturbances in B-deficiency unless the rabbit heart is entirely different from that of the rat and man.

It will be seen from Fig. 2 that the rabbit metabolizes 95 % of the injected pyruvate during the time of injection (2-3 min.). There is a steady fall of blood pyruvate during the first 5 min. until the level reaches that which is found in the deficiency states (1-2 mg./100 g.), then the fall is very slow. These experiments show that a normal rabbit can metabolize 100-150 mg. or more pyruvate in 20 min.

From this it seems to follow that increased blood pyruvate cannot be the direct cause of bradycardia, and hence possibly not of any cardiac disfunction. It is more likely that the two phenomena have some common causative factor.

Since the preliminary communication of the results in this paper was made [Lu, 1938] a paper appeared by Kalaja & Närvänen [1938] in which the conclusion is reached from the effects of subcutaneous injections of various metabolites

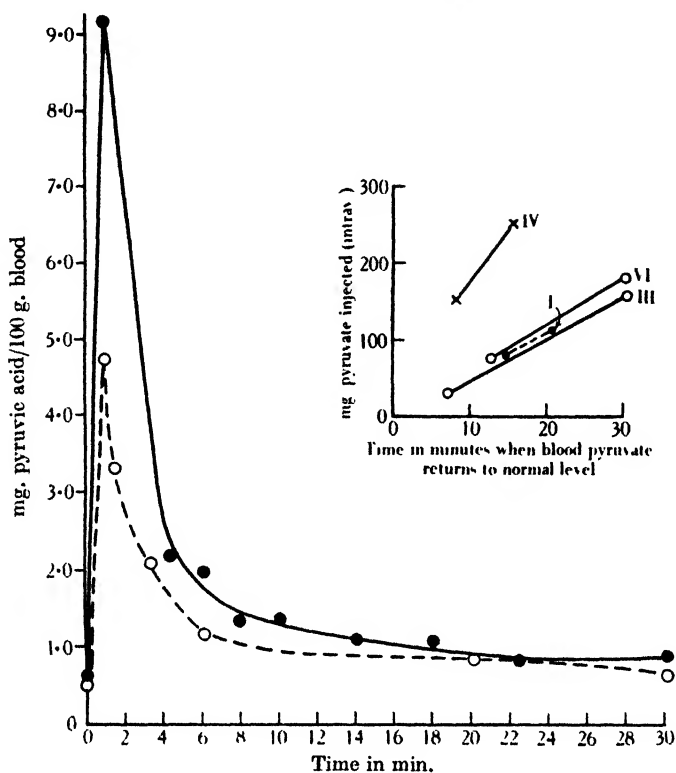


Fig. 2. Pyruvate of normal rabbits after injection. ●—● Curve I. 250 mg. pyruvate injected (2½ min.). ○ - - ○ Curve II. 160 mg. pyruvate injected (2 min.).

which are known to accumulate in B-deficiency on the heart rates of rats, pigeons and rabbits, that the rat heart is singularly susceptible to various compounds, and that pyruvic acid has a powerful effect. 100–150 mg. were the smallest amounts to cause a definite and regular change of heart rate. An example was given of one rat which had 300 mg. of pyruvate and showed a gradual slowing of the heart rate lasting from 30 min. to 3 hr. after injection. These authors also state that in spite of the heavy subcutaneous dose the ketonic acid content of the blood increased relatively little during the experiment. As this finding has a special bearing on the specific relation of the biochemical lesion produced by B<sub>1</sub>-deficiency to the functional change of the heart, the following groups of rats were given subcutaneous injections of different doses and samples of Na pyruvate. Typical data for the cardiac and blood changes are illustrated in Table IX.

The examples cited in Table IX illustrate several important points, the first of which is concerned with pyruvic acid itself. Lipschitz *et al.* [1938] and Peters [1936] observed the toxic effects of pyruvate polymerides on tissues in their *in vitro* experiments. Since the same concentration neutralized under proper conditions when injected gives rise to no toxic symptoms, this fact may in part explain Kamiya's [1937] observations of the action of pyruvic acid on blood vessels.



Table IX

Groups	Amount of pyruvic acid injected as Na salt mg.	Time after the injection when sample obtained min.	Change of heart rate per min.	Blood pyruvate mg./100 g.	B.S.S.* expressed as pyruvic acid mg./100 g.	Remarks
I <sub>1</sub>	180	10	- 45	4.6	17.08	All rats of this group had immediate reactions of shivering which later developed into convulsions. 30 % of the injected rats died within 20 min.
I <sub>2</sub>	180	30	- 105	7.2†	34.16	
I <sub>3</sub>	180	60	- 200	10.0†	59.47	
II <sub>1</sub>	170	5	+ 20 nil	12.6	—	Convulsions at the end of the experiment when the tail is touched
II <sub>2</sub>	68	60	+ 25 nil	9.1	—	
II <sub>3</sub>	34	60	+ 10 nil	5.2	—	
II <sub>4</sub>	102	120	- 175	11.4	—	
II <sub>5</sub>	68	120	- 15 nil	6.4† trace	—	
III <sub>1</sub>	75	5	+ 20 nil	11.3	18.9	Convulsion on touching the tail after 1½ hr.
III <sub>2</sub>	30	10	+ 40	24.0	36.2	
III <sub>3</sub>	30	120	± 0 nil	9.8	14.7	
III <sub>4</sub>	75	120	- 130	14.8†	62.0	

\* B.S.S. was not estimated in samples from group II.

† These samples showed intense purple colour of methylglyoxal bishydrazone with alcoholic KOH when tested by the method of Barrenscheen & Dreguss [1931]. Group I. The pyruvic acid solution used was prepared by neutralizing a 1 : 3 dilution of twice freshly redistilled pyruvate to pH 7.4 with 20 % NaOH; this contains 180 mg. per ml. Group II. B.D.H. Na pyruvate, which has 85 % of its B.S.S. as pyruvate. Group III. Pyruvic acid used was prepared by diluting the twice redistilled pyruvic acid and neutralizing to pH 7.4. Just before use it was so diluted that 1 ml. contained 100 mg.

When a pyruvic acid solution neutralized in the concentrated form was diluted and injected into rabbits it produced tremor and vasoconstriction. Examples given in Table IX demonstrate the toxic effect of such preparations in living rats. Since the whole musculature of the rats was involved, the change of heart rate cannot be regarded as due to the specific effect of pyruvate. Furthermore there exists also a much greater proportional change of B.S.S. which also may exert a profound effect. The second interesting point is that the blood pyruvate level can be raised to 2 or 4 times that which was found in B<sub>1</sub>-deficiency lasting for 1 or 2 hr. (II<sub>2</sub>, III<sub>2</sub> and III<sub>3</sub>) without affecting the heart rate. Yet larger doses, lasting for longer periods (compare II<sub>1</sub> and II<sub>4</sub>; III<sub>1</sub> and III<sub>4</sub>) with practically no difference in blood pyruvate value, had a marked effect on the heart. As I have mentioned above, if the heart change is a chemical effect it should be transient and should respond rapidly to definite levels of pyruvate concentration in the fluid in which the heart is bathed.

## DISCUSSION

The *in vitro* experiments of Weil-Malherbe [1937] using brain tissue and Lipschitz *et al.* [1938] using liver and kidneys clearly show that removal of added pyruvate involves other substrates. Wilkins *et al.* [1938] have evidence that pyruvic acid is converted into other ketonic substances by blood both *in vitro* and *in vivo*. My own results, obtained from blood of rabbits and rats, also show that pyruvate is quickly converted into other bisulphite-binding compounds. In some

instances where the B.B.S. value occupied a dominating place in the blood changes, a methyglyoxal-like substance was obtained. In view of the above-mentioned facts it is obvious that pyruvate itself, in the amounts in which it occurs in vitamin B<sub>1</sub>-deficiency, has no effect on the rat heart. The slowing of heart rate produced by excessive doses (above 100 mg. for a rat weighing 50 g.) should be interpreted as due to secondary changes caused by the efforts of the organism to remove such an unexpected load of pyruvate.

The experiments reported in these two papers with rats suffering from B<sub>1</sub>-hypovitaminosis uncomplicated by any other condition, in which a close correlation was shown to exist between blood pyruvate level and heart rate, demonstrate that these changes are causally associated with B<sub>1</sub>-deficiency.

At the same time it is clear that the pyruvate and cardiac changes may be complicated and even obscured by other concurrent conditions. In any given case in man, only the combination of blood pyruvate measurements, exercise tolerance tests and clinical symptoms can reliably differentiate between true vitamin B<sub>1</sub>-deficiency and other conditions which in some respects may simulate it. The very rapid return to normality, both of the pulse rate and the blood pyruvate, when the vitamin is given to acute cases, strongly supports the view that the lesion there is biochemical or "functional". If the deficiency is allowed to continue until "organic" or histological changes have set in, then administration of the vitamin may remove the acute symptoms by restoring the blood pyruvate to the normal level, but will not reverse the cardiac changes. If, however, blood pyruvate levels raised many times above the concentration found in deficiency states in rabbits and rats give rise to no change of heart rate. The raised pyruvate levels can hardly, therefore, be the direct cause of the heart rate changes. On the other hand, the results reported in these papers support the view that the pyruvate test is specific for B<sub>1</sub>-deficiency, presumably because the deranged metabolism of this deficiency involves the accumulation of pyruvic acid in the blood, and the test can be applied for assessing B<sub>1</sub>-deficiency in man.

#### SUMMARY

1. Blood pyruvate values for normal and avitaminous animals are presented.
2. Blood pyruvate and bradycardia run parallel in rats suffering from vitamin B<sub>1</sub>-deficiency.
3. Injection of pyruvate into the circulation of normal rats and rabbits, however, even above the highest levels occurring in vitamin B<sub>1</sub>-deficiency, has no effect on the heart rate.
4. It is unlikely therefore that the raised blood pyruvate in B<sub>1</sub>-deficiency is the direct cause of the cardiac symptoms; it is itself rather the result of the metabolic derangements characterizing this condition.
5. This is supported by the fact that blood pyruvate in man may reach the normal level long before the pulse rate does so.
6. Even moderate muscular exercise considerably increases the blood pyruvate.

The author is indebted to the Henry Lester Institute of Medical Research, Shanghai, for a travelling Fellowship and to Sir F. G. Hopkins for the hospitality of his Institute. She also wishes to thank Dr L. J. Harris for facilities and help at the Nutrition Institute in the case of some of the rat experiments.

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# XCIX. ISOLATION AND PROPERTIES OF A FLAVOPROTEIN FROM HEART MUSCLE TISSUE

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*(Received 30 March 1939)*

SINCE Warburg & Christian [1932] isolated the first flavoprotein from yeast (flavinphosphoric acid as its prosthetic group) and Banga & Szent-Györgyi [1932] observed the presence of flavin in muscle tissue, there has been much speculation on the possible role of a flavoprotein in the mechanism of oxidations in animal tissues. As yet, however, there is no information on the state and function of flavoprotein in animal tissues.

I have purified from heart muscle tissue a flavin compound, the coenzyme of the *d*-amino-acid oxidase, which is neither free flavin, nor flavinphosphoric acid [Straub, 1938]. Warburg & Christian [1938, 1] at the same time isolated the same compound and later [Warburg & Christian, 1938, 2, 3] described it as a flavinadenine dinucleotide. I have pointed out that in muscle tissue the dinucleotide is attached to some other protein than that of *d*-amino-acid oxidase. In the present work I shall describe the isolation and properties of a flavoprotein from heart muscle tissue,<sup>1</sup> which has this flavinadenine dinucleotide as its prosthetic group. In a preliminary note it has been already reported that this flavoprotein is the physiological agent for the oxidation of cozymase in animal tissues; it is in fact identical with the so-called diaphorase or coenzyme factor [Straub *et al.* 1939].

## *Catalytic test*

The concentration of the flavoprotein during the process of purification was followed with the help of the *d*-amino-acid oxidase test. The O<sub>2</sub> uptake was measured in Barcroft manometers containing coenzyme-free *d*-amino-acid oxidase [Straub, 1938] and *dl*-alanine, at 38° in phosphate buffer of pH 7.4. When flavinadenine dinucleotide is added to this mixture, it will combine with the protein to form *d*-amino-acid oxidase, thus oxidizing the alanine. The velocity of the oxidation is proportional to the amount of dinucleotide added.

If, instead of dinucleotide, a flavoprotein, which has the dinucleotide as its prosthetic group, is added to the test, no O<sub>2</sub> uptake is observed. If the flavoprotein solution is first heated in a boiling water bath for 3 min., the protein becomes denatured, thus liberating free dinucleotide. If this "boiled" solution is added to the test, the O<sub>2</sub> uptake will indicate the amount of dinucleotide, i.e. the amount of flavoprotein which was present in the solution.

The correlation between O<sub>2</sub> uptake and concentration of dinucleotide in the test solution has been studied by Warburg & Christian [1938, 3]. They find that

$$c = k \cdot \frac{x}{s - x},$$

where *k* is a constant, *x* is the O<sub>2</sub> uptake in 10 min. in a test containing *c* mol. dinucleotide per litre and *s* is the O<sub>2</sub> uptake in 10 min. in a test, which contains

<sup>1</sup> Preliminary note [Straub, 1939].

the same amount of *d*-amino-acid oxidase as the former and an excess of dinucleotide. As this formula is valid only for one particular concentration of *d*-amino-acid oxidase, I have used it in a different form:

$$c = k \cdot \frac{s-x}{x} \cdot \frac{s}{100},$$

where *c*, *s* and *x* have the same meanings as before. The factor 100 is introduced only to give *k* a practicable value, namely, the concentration of dinucleotide in the test solution (mol./l.) which gives 50  $\mu$ l. O<sub>2</sub> uptake in 10 min., when the same amount of enzyme with an excess of dinucleotide gives 100  $\mu$ l. O<sub>2</sub> absorption in 10 min. The modified formula gives the same value of *k* for different values of *s*, i.e. for different amounts of *d*-amino-acid oxidase, within reasonable limits. The value of *s* in the present work was usually 100–150  $\mu$ l. O<sub>2</sub> in 10 min. The value of *k* in this formula was found to be  $2.37 \times 10^{-7}$  under the experimental conditions mentioned above, and with air in the gas space of the manometers. Table I gives the results of one such test.

Table I

Manometer no. ...	1	2	3
Coenzyme-free <i>d</i> -amino-acid oxidase (ml.)	1.0	1.0	1.0
Purified dinucleotide solution (50 $\mu$ g. lactoflavin/ml.)	—	—	1.0
Heated flavoprotein solution (0.047 mg. protein/ml.)	—	1.5	—
4.5 % <i>dl</i> -alanine solution	0.3	0.3	0.3
Phosphate buffer	2.0	0.5	1.0
$\mu$ l. O <sub>2</sub> uptake in 10 min. at 38°	0	67.3	139
$\mu$ g. lactoflavin in form of dinucleotide	—	0.39	—
% lactoflavin in flavoprotein	—	0.57	—

Spectrophotometrically found: 0.54% lactoflavin.

The fact that purified flavoprotein solutions, when added to the test, do not give rise to any O<sub>2</sub> absorption, means that the dinucleotide has a much greater affinity towards the protein of the flavoprotein than towards the protein of the *d*-amino-acid oxidase. If in the test a *d*-amino-acid oxidase is used which is not completely free from its coenzyme and consequently still shows some O<sub>2</sub> absorption without any addition of dinucleotide, this O<sub>2</sub> uptake will diminish on addition of a purified flavoprotein solution. Thus it appears that a small fraction (0.5–1 %) of the flavoprotein has lost its prosthetic group during the purification and this free protein is able to bind flavindinucleotide at the expense of the *d*-amino-acid oxidase.

When the flavoprotein is heated during the purification, part of it becomes denatured, and the solution will therefore contain free dinucleotide as well as flavoprotein (bound dinucleotide). For this reason a control experiment was carried out on each occasion, adding in one test the flavoprotein solution before, and in the other after, boiling. The first result gives the amount of free dinucleotide, the second the sum of free and bound forms; the difference represents the amount of undenatured flavoprotein.

From the nature of the test it appears that any protein which has the dinucleotide as its prosthetic group would be included in the determination. The purification however yields only one flavoprotein, which behaves uniformly. There is no indication that any other similar flavoprotein except that described is present in the solutions obtained.

*Purification*

Eleven pigs' hearts are freed from connective tissue and fat and then minced. The muscle tissue is washed 3 times with 15–20 vol. tap water for 20 min. each time with vigorous mechanical stirring. The water is pressed out through a thin cloth.

1660 g. of the washed mince are ground with 2.5 l.  $M/50$   $\text{Na}_2\text{HPO}_4$  solution and about 500 g. sand in a mechanical mortar for 1–1½ hr.<sup>1</sup> The pulp is then thoroughly mixed with 2.5 l. distilled water and centrifuged.

The 4.7 l. of supernatant suspension (A) are mixed with 118 ml.  $M$  acetate buffer ( $pH$  4.6) and centrifuged. The precipitate is suspended in 1660 ml. distilled water to which 33 g.  $(\text{NH}_4)_2\text{SO}_4$  and 50 ml. ethyl alcohol are added and the mixture is heated on a water bath to 43° for 10–15 min. The insoluble muscle proteins are thus denatured and at the same time the flavoprotein becomes dissolved. When centrifuged, 1370 ml. of a somewhat opalescent strong yellow solution containing the enzyme (B) are obtained.

The solution is cooled down and then mixed with 45 ml. of an aluminium hydroxide ( $\text{C}_\gamma$ ) gel. (1.0 g.  $\text{Al}_2\text{O}_3$ ). After ½ hr. the alumina is centrifuged down. The solution is then mixed with a further 22 ml. alumina and again centrifuged. The enzyme is absorbed on the alumina and it can be eluted with alkaline phosphate. With successive lots of 50 ml.  $M/5\text{Na}_2\text{HPO}_4$  solution the elution is carried on until the eluate appears to be colourless. The eluates are combined, making altogether 240 ml., and dialysed against distilled water until salt-free (C).

The precipitate, which is formed during the dialysis, is not removed but it will redissolve on addition of 2.8 g.  $(\text{NH}_4)_2\text{SO}_4$  to the 280 ml. dialysate. The solution is now heated on a water bath for 5 min. to 60° and centrifuged after it has cooled down. The precipitate is discarded and 76 g. solid  $(\text{NH}_4)_2\text{SO}_4$  is added to the solution (0.45 sat.). After standing in the ice chest for 1 hr., the precipitate is filtered off<sup>2</sup> and the solution (285 ml.) is mixed with 71 g.  $(\text{NH}_4)_2\text{SO}_4$  (0.8 sat.). The precipitate is filtered off and the solution discarded. The yellow precipitate is washed from the filter paper with distilled water in about 30 ml. and dialysed overnight against 1.5 l. distilled water. The precipitate thus formed contains no enzyme, it is centrifuged off and discarded. The solution is then further dialysed against fresh distilled water. The precipitate which is now formed is usually yellow and contains some enzyme. It is not removed, but the dialysis continued until the solution is salt-free (D).

0.1 %  $\text{NH}_3$  is added drop by drop to the dialysate until the precipitate dissolves. The solution is now heated for 5 min. to 60–62° without any salt addition and the precipitate centrifuged off. 10.9 g.  $(\text{NH}_4)_2\text{SO}_4$  are added to the 35 ml. solution (0.5 sat.) and the precipitate filtered off and discarded. From the 40 ml. filtrate the enzyme is precipitated by adding 4 g.  $(\text{NH}_4)_2\text{SO}_4$  (0.65 sat.). The yellow precipitate is filtered off and washed from the filter with distilled water. The solution thus obtained (15 ml.) is dialysed thoroughly against distilled water. A small yellow precipitate is formed, which is centrifuged off, leaving 20 ml. of the flavoprotein solution (E). It is advisable to add a few drops of dilute alkali or phosphate, when it will remain clear.

<sup>1</sup> The time of grinding depends on the type of the mortar and cannot therefore be definitely stated. It is carried on until a small sample shows, after centrifuging, only a thin layer of myosin above the layers of sand and heavier muscle tissue lumps. If the grinding is continued until the whole mass becomes a uniform paste, too much myosin is extracted which cannot properly be centrifuged down, so that the supernatant suspension will be only half the volume of the added solutions, thus reducing the yield.

<sup>2</sup> All the filtrations have been done with Whatman no. 1 filters. No suction can be applied.

Table II. *Yield and flavin content during the purification*

	Protein dry weight g.	mg. lactoflavin present as protein-bound dinucleotide	Yield of bound dinucleotide %	Lacto-flavin %
1660 g. washed muscle	320	6.9	(100)	0.0022
(A) Phosphate extract	39	2.8	42	0.007
(B) Alcohol-(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> solution	—	0.97	14	—
(C) Alumina eluate	1.05	0.65	9.4	0.002
(D) First (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation	0.27	0.56	8.1	0.21
(E) Second (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation	0.098	0.53	7.6	0.54*

\* Spectrophotometrically determined.

*Properties of the flavoprotein*

The colour of the flavoprotein solution is yellow, although it appears greenish because of the strong greenish fluorescence. The spectrum of the flavoprotein<sup>1</sup> is reproduced in Figs. 1 and 2. It shows maxima at 274, 359 and 451 m $\mu$  and minima at 250, 316 and 398 m $\mu$ . When examined under the low-dispersion microspectroscope, two distinct bands are seen in the visible region of the spectrum, one at 480–490 m $\mu$ , the other at 440–460 m $\mu$ . The existence of these bands in muscle extracts has been previously observed by Keilin & Hartree [1939].

From the absorption at 451 m $\mu$  the lactoflavin content of the purified preparations has been determined, by assuming that the value for this maximum is the same as in any other flavoprotein, i.e.  $\beta_{641} = 2.4 \times 10^7$ . The flavin content thus found in the purest preparations is  $0.54 \pm 0.02$  % lactoflavin. (Expressed as lactoflavinphosphate 0.66 %.) The molecular weight calculated from the flavin content is  $\frac{100 \times 376}{0.54} = 70,000$ .

That the flavinadenine dinucleotide is the prosthetic group of the flavoprotein is shown by the fact that it can quantitatively replace the coenzyme of the *d*-amino-acid oxidase. No attempt has been made to isolate the prosthetic group from the purified enzyme.

The flavoprotein is reduced to the leuco compound by Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> and it is reoxidized when shaken with air. It is also reduced to the leuco form by dihydrocozymase.

The fluorescence of the flavoprotein solutions is just as strong as that of the free flavin. It is not due to the presence of free flavin and it is completely extinguished on the addition of reduced cozymase. The old yellow enzyme of Warburg & Christian does not show any fluorescence. The difference might be explained if the alloxazin ring is not bound to the protein in the case of the heart flavoprotein as it is in the case of the old yellow enzyme [Kuhn & Boulanger, 1936]. This supposition is supported by the fact that the absorption band of the flavoprotein coincides with the bands of the free flavin.

The flavoprotein is in an insoluble state in the tissue, but this is not an intrinsic property of the enzyme. During the purification, when heated with 3 % alcohol, the enzyme is detached from the insoluble muscle proteins and becomes soluble in salt solution. After removing impurities it is soluble even in salt-free solutions.

The N content of the purified flavoprotein was found to be 15.7 %.

<sup>1</sup> The spectrum was taken with a Hilger "Spekker" apparatus. I am indebted to Mr H. S. Corran for carrying out the measurements.

The heat stability of the flavoprotein is remarkable, and it increases during the purification. When it is already in a soluble form, heating for 5 min. to 60° does not cause any destruction, while heating for 5 min. to 70° only destroys

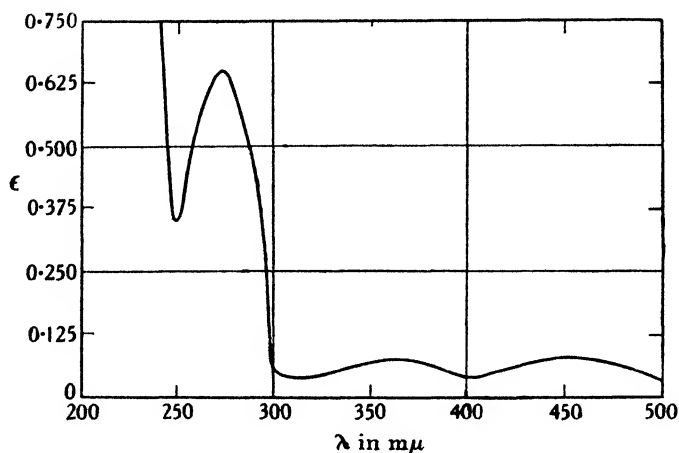


Fig. 1. Absorption spectrum of a 0.0265% solution of the flavoprotein ( $d=2$  cm.).

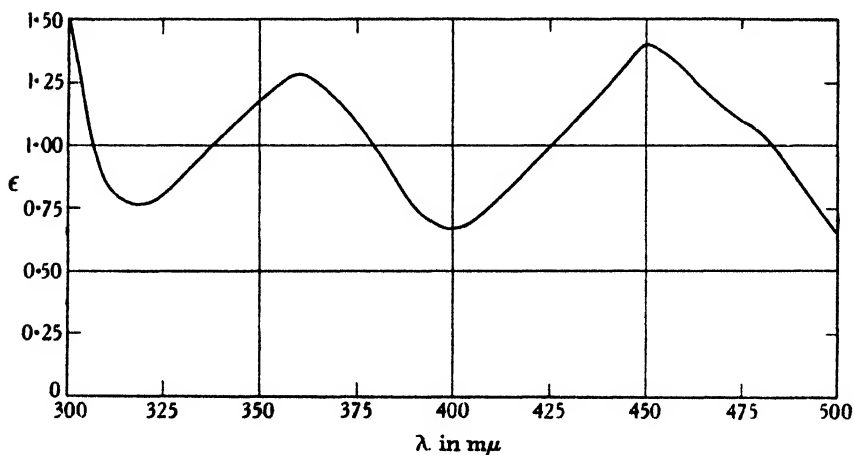


Fig. 2. Absorption spectrum of a 0.47% solution of the flavoprotein ( $d=2$  cm.).

about 10%. The heat stability of the purified preparation will be discussed in a subsequent paper.

I wish to express my deep gratitude to Prof. D. Keilin for his constant help and interest in this work.



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# C. ON THE CATALYTIC FUNCTION OF HEART FLAVOPROTEIN

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THE oxidation in animal tissues of metabolites such as lactate, malate,  $\beta$ -hydroxybutyrate, triosephosphate etc., can be resolved into at least three consecutive reactions:

- (1) Metabolite + coenzyme I  $\rightarrow$  oxidized metabolite + reduced coenzyme I.
- (2) Reduced coenzyme I + carrier  $\rightarrow$  coenzyme I + reduced carrier.
- (3) Reduced carrier +  $O_2 \rightarrow$  carrier.

The first reaction is catalysed by a family of enzymes, each of which is specific for a particular metabolite. For example, the lactic enzyme catalyses the oxidation of lactate by coenzyme I; the malic enzyme the oxidation of malate, etc. There are three components in this reaction, viz. enzyme, metabolite and coenzyme I, and the sole chemical change is the catalysed transfer of hydrogen from the metabolite to the coenzyme. The second reaction is not spontaneous, but requires a special catalyst, referred to as coenzyme factor or diaphorase [Euler & Hellström, 1938; Dewan & Green, 1938]. The mechanism whereby the coenzyme factor catalysed the oxidation of reduced coenzyme was obscure owing to lack of information concerning the chemical nature of the catalyst. Recently Straub [1939, 1, 2] isolated from heart muscle a highly purified flavoprotein compound with properties which at once suggested its identity with the coenzyme factor. This flavoprotein is rapidly reducible by dihydrocoenzyme I and its leuco- or reduced form is rapidly oxidizable by suitable carriers. Reaction (2) therefore can be further resolved into two constituent processes:

- (2a) Reduced coenzyme I + flavoprotein  $\rightarrow$  coenzyme I + leucoflavoprotein.
- (2b) Leucoflavoprotein + carrier  $\rightarrow$  flavoprotein + reduced carrier.

To complete our knowledge of how certain metabolites are oxidized by molecular oxygen via coenzyme I, it will be necessary to know what substance or substances in animal tissues can assume the role of carrier. In reconstructed systems artificial carriers such as methylene blue are employed. There is evidence that the cytochromes are the physiological equivalents of methylene blue, but the possibility remains that the hypothetical reaction between dihydrocoenzyme and the cytochromes is complex.

## I. CATALYTIC PROPERTIES OF HEART FLAVOPROTEIN

For the following experiments a preparation of heart flavoprotein at the 0.66% flavinphosphate level of purity was used (cf. Straub [1939, 2] for the details of the method of isolation and purification). The concentration of the flavoprotein in solution was estimated spectrophotometrically from the light absorption at  $450\text{ m}\mu$ , the absorption coefficient at  $450\text{ m}\mu$  being taken as  $2.4 \times 10^7$ . The concentration of flavoprotein was also estimated by an independent method which

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involves splitting off the prosthetic group (adenineflavin dinucleotide) and estimating the concentration of the prosthetic group in the amino-acid oxidase test system. We shall consider this method in greater detail in section III. Both methods of estimating the concentration of flavo-protein agreed to within *ca.* 5%.

Reduced coenzyme I is oxidized extremely slowly by methylene blue. In presence of heart flavoprotein rapid reaction takes place. The reduced coenzyme-methylene blue system thus offers a convenient "test" for measuring quantitatively the catalytic action of heart flavoprotein. Dihydrocoenzyme was prepared either chemically, by reducing coenzyme with hydrosulphite and removing excess hydrosulphite by aeration, or enzymically by reducing coenzyme with the lactic enzyme-lactate system. The velocity of the catalytic oxidation of dihydrocoenzyme I by methylene blue was measured either anaerobically by the rate of decoloration of methylene blue or aerobically by the rate of O<sub>2</sub> absorption (*cf.* reactions (2) and (3)).

Table I summarizes a manometric experiment designed to show the catalytic effect of heart flavoprotein. The lactic enzyme was prepared from rabbit skeletal muscle by the method of Green *et al.* [1937]. The function of cyanide is to trap

Table I. *Catalytic effect of flavoprotein in the lactic enzyme system*

Lactic enzyme (ml.)	1.5	1.5	1.5	1.5	1.5
M lactate (ml.)	0.2	—	0.2	0.2	0.2
0.075% coenzyme I (ml.)	1.0	1.0	—	1.0	1.0
2 M HCN (ml.)	0.2	0.2	0.2	0.2	0.2
0.5% methylene blue (ml.)	0.2	0.2	0.2	—	0.2
Flavoprotein (ml.) (17.5 µg./ml. as flavinphosphate)	0.1	0.1	0.1	0.1	—
Water	—	0.2	1.0	0.2	0.1
µl. O <sub>2</sub> in 5 min.	340	14	14	14	14

the product of oxidation, viz. pyruvate, which inhibits the forward reaction. For the oxidation of lactate by molecular O<sub>2</sub> both flavoprotein and methylene blue are necessary. The fact that there is no appreciable O<sub>2</sub> uptake in absence of methylene blue indicates that reduced flavoprotein is not autooxidizable under the conditions of the experiment. Increase of the flavoprotein concentration beyond *ca.* 0.5 µg. flavinphosphate equivalent per 3.6 ml. does not increase the rate of O<sub>2</sub> uptake. The effect of successive dilution of flavoprotein on the rate of O<sub>2</sub> uptake is shown in Table II. A measurable catalytic effect is given by

Table II. *Variation of O<sub>2</sub> uptake of lactic system with flavoprotein concentration*

The complete system contained 1.5 ml. enzyme, 0.2 ml. M lactate, 1 ml. 0.075% coenzyme I, 0.2 ml. 2 M HCN and 0.2 ml. 0.5% methylene blue. Total vol. 3.6 ml., 38°, air in gas space.

Flavoprotein in µg. flavin- phosphate	µl. O <sub>2</sub> /5 min.
1.74	385
0.87	385
0.53	360
0.35	267
0.18	160
0.087	90
0.053	70
0.018	37
0	2

0.005 µg. flavinphosphate equivalent/ml. The homogeneous flavoprotein contains 0.66% flavinphosphate. 0.018 µg. of flavinphosphate therefore is equi-

valent to 2.77  $\mu\text{g.}$  of flavoprotein. Since 2.77  $\mu\text{g.}$  catalyse the absorption of 444  $\mu\text{l. O}_2/\text{hr.}$  the  $Q_{O_2}$  ( $\mu\text{l. O}_2/\text{hr./mg.}$ ) is 160,000. In terms of the flavinphosphate moiety the  $Q_{O_2}$  is *ca.*  $2.5 \times 10^7$ . Fig. 1 shows the dependence of the turnover number (T.N.) on the concentration of flavoprotein. The formula for calculating T.N. is

$$\frac{\mu\text{l. O}_2/\text{min.}}{0.049 \times \mu\text{g. flavinphosphate}}$$

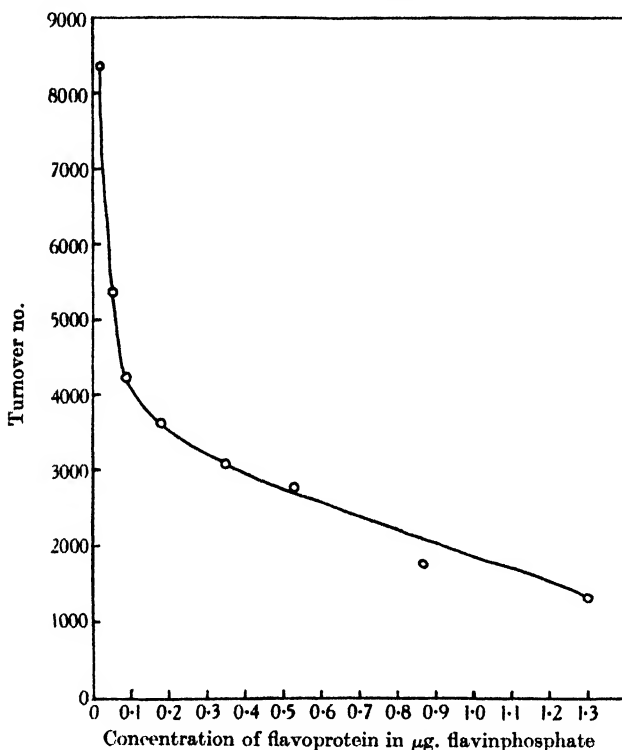


Fig. 1. Dependence of turnover number on flavoprotein concentration. Details as in Table I.

Strictly speaking the numerator should be  $\mu\text{l. H}_2/\text{min.}$  which is  $2 \times \mu\text{l. O}_2/\text{min.}$  But since in the autoxidation of methylene blue an equivalent amount of  $\text{H}_2\text{O}_2$  is produced, 1  $\mu\text{l. O}_2$  absorbed is equivalent to 1  $\mu\text{l. H}_2$  transferred. The limiting value of the T.N. is *ca.* 8000/min.

Comparison of the catalytic activities of the heart and Warburg-Christian yeast flavoprotein shows a tremendous difference. For example, 0.015  $\mu\text{g.}$  flavinphosphate equivalent of heart flavoprotein catalysed the same  $\text{O}_2$  uptake as 5.3  $\mu\text{g.}$  flavinphosphate equivalent of Warburg-Christian flavoprotein. The ratio of activities was therefore  $5.3/0.015 = 353$ .

The flavoprotein effect is independent of the nature of the enzyme which is concerned in the reduction of the coenzyme. Table III shows that by replacing the lactic enzyme-lactate system by the malic, triosephosphoric or  $\alpha$ -glycerophosphoric enzyme systems, the same qualitative results obtain. The quantitative differences are referable to the activities of the different enzymes and the speed with which dihydrocoenzyme is made available by the various enzyme systems.

Table III. *Catalytic effect of heart flavoprotein in the malic, triosephosphoric and  $\alpha$ -glycerophosphoric enzyme systems*

The dialysed extract of the acetone powder of rabbit skeletal muscle was used as the source of the three enzymes. Cyanide was used as fixative in the malic and  $\alpha$ -glycerophosphoric enzymes. For further experimental details, cf. Green *et al.* [1937]. The enzyme system in all cases contained enzyme, substrate, coenzyme I and methylene blue. The amount of flavoprotein added was 2  $\mu$ g. flavinphosphate equivalent.

	$\mu$ l. O <sub>2</sub> in 5 min.
Malic enzyme system	2
Malic enzyme system + flavoprotein	168
$\alpha$ -Glycerophosphoric enzyme system	2
$\alpha$ -Glycerophosphoric enzyme system + flavoprotein	138
Triosephosphoric enzyme system	2
Triosephosphoric enzyme system + flavoprotein	60

A solution of dihydrocoenzyme I was prepared according to the method of Green & Dewan [1937]. Spectrophotometric estimation showed that the solution contained 1900  $\mu$ g. dihydrocoenzyme per ml. 2 ml. of this solution reduced 0.15 ml. of 0.125 *M* methylene blue in 10 min. In presence of 3  $\mu$ g. flavinphosphate equivalent of flavoprotein the reduction time was 10 sec. It was of theoretical interest to compare the T.N. of flavoprotein in this system with those obtained in the lactic enzyme system. The following figures show the T.N. for 0.13 and 0.03  $\mu$ g. flavinphosphate equivalent of flavoprotein in the two systems.

	Turnover number	
	Hypsulphite reduced coenzyme	Enzymically reduced coenzyme
0.13 $\mu$ g. flavinphosphate	3700	3850
0.03 $\mu$ g. flavinphosphate	6200	6250

The correspondence is remarkably close and offers convincing proof that the enzyme system has no influence on the catalysed reaction between dihydrocoenzyme and methylene blue except in so far as it regulates the velocity with which dihydrocoenzyme I is formed.

Some anaerobic experiments have been carried out on the catalysed reaction between methylene blue and dihydrocoenzyme I prepared *in situ* by the lactic enzyme system. Table IV summarizes a controlled experiment. Increase of the flavoprotein concentration beyond 1  $\mu$ g. flavinphosphate equivalent does not increase the rate of reduction of methylene blue. In other words, above 1  $\mu$ g. flavinphosphate equivalent the limiting factor under the conditions of the experiment is the enzymic rate of formation of dihydrocoenzyme and not the concentration of flavoprotein. We should expect on theoretical grounds that the

Table IV. *Flavoprotein effect in the lactic enzyme system under anaerobic conditions*

The experiment was carried out in evacuated Thunberg tubes at 38°. The complete system contained 1 ml. enzyme, 0.5 ml. 0.075 % coenzyme I, 0.2 ml. 2 *M* HCN, 0.2 ml. 0.0125 *M* methylene blue and 0.1 ml. flavoprotein solution containing 17.5  $\mu$ g. flavinphosphate per ml.

	Reduction time
Complete system	45 sec.
Complete without flavoprotein	$\infty$
Complete without lactate	$\infty$
Complete without coenzyme	$\infty$

Table V. *Turnover number of flavoprotein in lactic system under anaerobic conditions*

Details as in Table IV.		
Flavoprotein concentration in $\mu\text{g. flavin-phosphate}$	Reduction time in min.	T.N.
3.50	0.75	435
1.75	0.75	870
0.87	1.08	1180
0.53	1.58	1360
0.35	3.33	975
0.18	10	625

efficiency of flavoprotein as a catalyst would progressively increase on dilution beyond the saturating concentration. Table V shows that the T.N. first increases and then falls with successive dilutions of flavoprotein. This fall in the T.N. can only mean destruction of flavoprotein under the conditions of the experiment. Below 0.05  $\mu\text{g. flavinphosphate}$  equivalent per ml. no catalytic effect can be demonstrated under anaerobic conditions, whereas aerobically a measurable effect can be obtained with 0.005  $\mu\text{g.}$  We have no explanation for the anaerobic destruction of flavoprotein.

Heart flavoprotein also catalyses the oxidation of dihydrocoenzyme II by methylene blue. The hexosemonophosphoric enzyme system was used as the reducing system for coenzyme II. The flavoprotein-free enzyme was prepared from rabbit skeletal muscle by the following unpublished method of one of us (H. S. C.). Acetone powder of rabbit skeletal muscle was mixed with water (2 vol.), stirred to a homogeneous paste for 20 min., and the mixture was strained through muslin. The filtrate was centrifuged and dialysed. Coenzyme II was prepared from horse red blood corpuscles by the method of Warburg & Christian [1933]. One of us (H. S. C.) has established that the hexosemonophosphoric enzyme of rabbit skeletal muscle specifically collaborates with coenzyme II. Table VI shows the catalytic effect of heart flavoprotein on the reaction between

Table VI. *Effect of flavoprotein on hexosemonophosphoric enzyme system*

Enzyme (ml.)	2.0	2.0	2.0	2.0
0.1% crude coenzyme II (ml.)	0.5	—	0.5	0.5
0.3 M hexosemonophosphate (ml.)	0.5	0.5	—	0.5
Flavoprotein (ml.) (17 $\mu\text{g. flavinphosphate/ml.}$ )	0.2	0.2	0.2	—
0.0125 M methylene blue (ml.)	0.1	0.1	0.1	0.1
M/2 phosphate buffer pH 7.2 (ml.)	0.3	0.3	0.3	0.3
Water	—	0.5	0.5	0.2
Reduction time of methylene blue in min.	4	$\infty$	$\infty$	$\infty$

dihydrocoenzyme II and methylene blue. The activity of the enzyme preparation is too low to permit a more extensive investigation of the kinetics of coenzyme II systems. We have not therefore pursued this line further.

## II. MECHANISM OF THE CATALYSIS

On addition of a comparatively minute amount of the lactic enzyme system (i.e. enzyme, lactate, cyanide and coenzyme I) to a concentrated solution of heart flavoprotein the yellowish green colour is discharged within a few minutes at 16°. If any of the components of the lactic system is eliminated no decoloration is observed. The reduced leuco form is slowly autoxidizable as shown by the fact that shaking with air gradually restores the original colour (within a minute). Hydrosulphite also bleaches the colour of heart flavoprotein. Shaking

with air restores the colour as soon as excess hydrosulphite is removed. There is an apparent discrepancy in that leucoflavoprotein prepared enzymically is sluggishly autoxidizable, whereas when prepared by hydrosulphite reduction it is practically instantaneously oxidized by molecular oxygen. Keilin & Hartree [1936], in their study of catalase, observed that hydrosulphite yields  $\text{H}_2\text{O}_2$  when oxidized by air. The production of  $\text{H}_2\text{O}_2$  might well explain the difference in the rates of autoxidation under the two sets of conditions. To decide the question whether flavoprotein is reduced and oxidized in the course of its catalysis the following experiment was carried out. Two Thunberg tubes were filled with 1.0 ml. of a flavoprotein solution containing 31  $\mu\text{g}$ . flavinphosphate equivalent and 0.5 ml. of the enzyme system (0.1 ml. purified enzyme, 0.1 ml. *M* lactate, 0.1 ml. 0.05 % coenzyme I and 0.1 ml. *M* HCN). One of the tubes also contained 0.05 ml. of 0.125 *M* methylene blue. The times for the anaerobic decoloration of flavoprotein and methylene blue were 6 and 61 min. respectively at 18°. If the catalytic action of heart flavoprotein involves a cycle of reduction by dihydrocoenzyme and oxidation by methylene blue, the ratio

$$\frac{\mu\text{l. H}_2 \text{ transferred to methylene blue}}{\mu\text{l. H}_2 \text{ equivalent of flavoprotein}}$$

should be equal to the ratio

$$\frac{\text{reduction time of methylene blue}}{\text{reduction time of flavoprotein}}.$$

The value of the first ratio was  $14/1.52 = 9.2$  and that of the second was  $61/6 = 10.2$ . Clearly the catalytic reduction of methylene blue can be entirely accounted for on the basis of the cyclical reduction and oxidation of flavoprotein.

Oxidized heart flavoprotein shows an intense greenish fluorescence on irradiation with ultraviolet light. Addition of the lactic enzyme system leads to the gradual disappearance of the fluorescence. Disappearance of fluorescence runs parallel with the formation of the reduced form. The fluorescence method is eminently suitable for following the reduction of heart flavoprotein.

The use of comparatively large concentrations of flavoprotein and minute amounts of the reducing enzyme system is merely a device to obtain a "slow motion" picture of the reduction of flavoprotein. Under physiological conditions the concentration of flavoprotein is minute compared with the concentration of coenzyme I. On mixing 30  $\mu\text{g}$ . flavinphosphate equivalent of flavoprotein with 190  $\mu\text{g}$ . of dihydrocoenzyme I at room temperature the yellow-green colour and green fluorescence of the oxidized form disappear almost instantaneously.

### III. IDENTITY OF HEART FLAVOPROTEIN AND COENZYME FACTOR

Coenzyme factor (diaphorase) is characterized by the following properties [cf. Euler & Hellström, 1938; Dewan & Green, 1938]. It occurs in association with insoluble particles from which it cannot be removed merely by washing the particles with water or salt solutions at neutral pH. Above 55° or in solutions below pH 4.6 and above pH 9 the enzyme is rapidly inactivated. Furthermore, fairly active and purified preparations of the enzyme seem not to contain any appreciable amounts of flavin. On the other hand heart flavoprotein is a soluble flavoprotein which is destroyed by temperatures not lower than 80°. It is also unstable below pH 4 and above pH 9. At first sight the resemblance between coenzyme factor and heart flavoprotein seems meagre, to say the least. In fact from the brief description of their respective properties one would be tempted to conclude that they were two distinct compounds. These differences however are more apparent than real.

The starting point of the isolation of heart flavoprotein is the standard preparation of coenzyme factor. That is to say, the flavoprotein is found initially associated with insoluble particles from which it cannot be removed by exhaustive washing with water. By exposure of this enzyme suspension to 2%  $(\text{NH}_4)_2\text{SO}_4$  and 3% ethyl alcohol at pH 4.6 and 43° the flavoprotein becomes separated from the particles (cf. Straub [1939, 2] for complete details) and behaves thereafter as a soluble protein. Significantly, when the flavoprotein is brought into solution the soluble extract shows very high coenzyme factor activity.

The prosthetic group of heart flavoprotein is flavinadenine dinucleotide. On boiling a solution of the flavoprotein the dinucleotide is liberated from the coagulated protein and can be estimated in the amino-acid test system. We should expect that if the coenzyme factor is identical with heart flavoprotein the ratio  $\frac{\text{coenzyme factor activity}}{\text{dinucleotide concentration}}$  would be of the same order of magnitude for both. The following is the protocol of a typical experiment. 0.5 ml. of a pig heart flavoprotein solution containing 1.64  $\mu\text{g}$ . flavinphosphate equivalent per ml. was found to have approximately the same activity in catalysing the oxidation of reduced coenzyme I by methylene blue (lactic enzyme system) as 0.5 ml. of a standard coenzyme factor preparation from pig heart. The respective  $\text{O}_2$  uptakes were 238 and 231  $\mu\text{l}$ .  $\text{O}_2$  in 5 min. When the same amounts of boiled flavoprotein and factor solutions were tested with the amino-acid oxidase the respective oxygen uptakes were 82 and 79  $\mu\text{l}$ .  $\text{O}_2$ . Taking the oxygen uptake in the lactic system as a measure of coenzyme factor activity and the  $\text{O}_2$  uptake in the amino-acid oxidase test as a measure of the dinucleotide concentration, the ratio

$$\frac{\text{coenzyme factor activity}}{\text{dinucleotide concentration}}$$

can be equated with the ratio

$$\frac{\mu\text{l. O}_2 \text{ in lactic system}}{\mu\text{l. O}_2 \text{ in amino-acid oxidase system}}$$

The respective ratios for coenzyme factor and flavoprotein were 2.92 and 2.90. Clearly there was sufficient flavoprotein in the heart factor preparation to account for all the catalytic activity shown in the lactic enzyme system.

The amino-acid oxidase was prepared free of flavin dinucleotide by the following modification of the method of Straub [1938]. Acetone powder of pig kidney was rubbed up with 10 vol. water and the mixture centrifuged after 20 min. standing. The supernatant was made 33% saturated with respect to  $(\text{NH}_4)_2\text{SO}_4$ , cooled to 0° and brought to pH 4.6. The precipitate was centrifuged and redissolved in water. This procedure was repeated twice. The extract of 10 g. was finally made up to 30 ml. in  $M/5$  phosphate buffer, pH 7.2. One ml. of this enzyme takes up ca. 150  $\mu\text{l}$ .  $\text{O}_2$ /10 min. at 38° in presence of excess flavinadenine dinucleotide (3  $\mu\text{g}$ . per manometric cup). In absence of dinucleotide there is a blank of ca. 20–30  $\mu\text{l}$ .  $\text{O}_2$ /10 min. which disappears after the enzyme preparation has been kept at 0° for about 24 hr. The enzyme is stable for weeks at 0° and can be kept free of bacterial infection by saturation with octyl alcohol. 1 ml. of the enzyme works at half the maximum rate in presence of ca. 1  $\mu\text{g}$ . of dinucleotide. For purposes of estimation the concentration of dinucleotide should be such as to cover the range from ca. 40 to 80  $\mu\text{l}$ .  $\text{O}_2$ /10 min. The following quantities were used: 1 ml. enzyme, 1 ml. buffer, pH 7.2 and 0.2 ml.  $M$  *dl*-alanine. The gas space contained air.

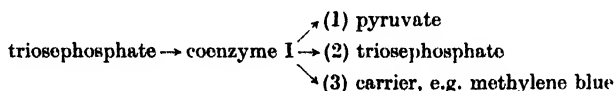
The inability of various investigators to detect flavin in the factor preparations of various tissues is easily explained by the following considerations. The standard coenzyme factor preparations contain on the average ca. 1.5  $\mu\text{g}$ . of flavinphosphate per ml. The method of detecting flavin involves treating the enzyme preparation with 3 vol. methyl alcohol and centrifuging off the denatured



protein. The supernatant fluid should be yellow if flavin is present. It is clear that even if all the flavin were split off by the methyl alcohol treatment, the concentration of flavin in the methyl alcohol solution (*ca.* 0.4  $\mu$ g. flavinphosphate per ml.) would be beyond the limit of visual detection. Heart flavoprotein solutions diluted so as to contain the same catalytic activity per ml. as the standard coenzyme factor preparations do not show any detectable colour and the presence of flavin dinucleotide is just detectable in the amino-acid oxidase test system.

The ratio  $\frac{\mu\text{l. O}_2 \text{ in lactic system}}{\mu\text{l. O}_2 \text{ in amino-acid oxidase system}}$  has been determined for coenzyme factor preparations from *Bact. coli*, rabbit kidney and liver. The respective ratios were 3.0, 2.8 and 1.9, as compared with a ratio of 2.9 for heart flavoprotein. The low value of the ratio for liver coenzyme factor preparations points to the presence of one or more additional flavoproteins which do not catalyse the oxidation of reduced coenzyme I. A coenzyme factor preparation made from yeast by the method of Dewan & Green [1938] was anomalous in that the amount of flavin dinucleotide was barely measurable although high activity was shown in the lactic enzyme test. This can mean either that the coenzyme factor of yeast is a flavoprotein with higher catalytic activity than that of animal tissues or that the active principle in the yeast preparations is not a flavoprotein.

Triosephosphate in presence of the aqueous extract of the acetone powder of rabbit skeletal muscle can undergo oxidation via three different mechanisms:



The arrows indicate the direction of transfer of hydrogen. In mechanisms (1) and (2) reduced coenzyme becomes oxidized by pyruvate in presence of the lactic enzyme or by triosephosphate in presence of the  $\alpha$ -glycerophosphoric enzyme. The net changes are in (1) the oxidation of triosephosphate by pyruvate, and in (2) the dismutation of triosephosphate to the corresponding alcohol and acid. In mechanism (3) reduced coenzyme I is oxidized by some carrier—the net change being the oxidation of triosephosphate by oxygen via the coenzyme and carrier. Whether mechanism (3) will be realized at the expense of (1) and (2) depends on the relative velocities with which reduced coenzyme I is oxidized in the different reactions. In presence of the coenzyme factor and methylene blue, it is possible to obtain a comparatively rapid reaction between triosephosphate and molecular O<sub>2</sub>. The Warburg-Christian flavoprotein shows practically no activity in the system even in high concentration. Presumably the coenzyme factor is able to catalyse the oxidation of reduced coenzyme I by methylene blue with a velocity of the same order of magnitude as that with which the lactic or glycerophosphoric enzymes catalyse the reduction of their respective substrates by dihydrocoenzyme I. In other words, the coenzyme factor must be as efficient a catalyst as the lactic and  $\alpha$ -glycerophosphoric enzymes in order to compete with them for the available dihydrocoenzyme I. The inactivity of the Warburg-Christian flavoprotein in the triosephosphoric system simply means that its catalytic efficiency is not high enough to compete with the other enzymes for dihydrocoenzyme. It is significant that heart flavoprotein can replace coenzyme factor in the triosephosphoric test system and shows the same order of catalytic efficiency.

Both coenzyme factor and heart flavoprotein agree in the following properties. They are both destroyed to the extent of *ca.* 50% by exposure to

solutions of pH 3.8 for 7 min. at 18°, and to the extent of ca. 90 % by exposure to solutions of pH 9.2 for 7 min. at 18°. At 55° and pH 7.0 a preparation of coenzyme factor loses 55 % of its activity after 10 min. exposure, whereas heart flavoprotein is half destroyed at about 85°. Were it not for the almost complete correspondence in other details between the properties of the two compounds, this formidable discrepancy in temperature lability could be used as definite proof of their non-identity. The coenzyme factor is associated with particles whereas the flavoprotein is soluble. Change in physical state might account for some difference in temperature-lability. It is also conceivable that the destruction of the coenzyme factor at 55° might be due to causes other than denaturation, e.g. hydrolysis by proteolytic enzymes associated with the insoluble particles. There is in fact evidence for this view. The instability of preparations of coenzyme factor extends even to temperatures as low as 38°. Exposure of the factor preparations of pig heart to 38° for 60 min. at pH 7.0 destroys some 30 % of their activity.

We may summarize the comparison of coenzyme factor and flavoprotein of heart as follows. The catalytic activity of both is proportional to the concentration of flavin dinucleotide. The respective catalytic activities are qualitatively and quantitatively identical. The starting point for the preparation of the soluble flavoprotein is the suspension of insoluble particles with which coenzyme factor is associated. Thus flavoprotein becomes soluble only as the result of special treatment of the suspension of insoluble particles. The two catalysts are destroyed below pH 4 and above pH 9 with equal velocities. They differ however in temperature-lability. On the whole the evidence is strongly in favour of the view that they are one and the same compound in two different physical states.

#### SUMMARY

Heart flavoprotein catalyses the oxidation of the dihydropyridinenucleotides by "carriers" such as methylene blue. The mechanism of the catalysis involves a cycle of reduction of the flavoprotein by dihydrocoenzyme and oxidation of its leuco form by the "carrier". Leucoflavoprotein is sluggishly autoxidizable. Under optimum conditions each molecule of flavoprotein catalyses the oxidation of 8500 molecules of dihydrocoenzyme I per min.

Coenzyme factor (diaphorase) is considered to be identical with heart flavoprotein. Whereas the former is found in association with insoluble particles, the latter has been separated from the particles and brought into solution.

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# CI. THE EFFECT OF PHLORIDZIN ON CARBOHYDRATE METABOLISM *IN VITRO*

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THE metabolic effect of phloridzin has been interpreted in several different ways. Some authors have claimed a solely renal action [Erlandsen, 1910; Allen, 1913] inasmuch as phloridzin affected the permeability of the renal tubules for glucose and other substances [Underhill, 1912], thus inhibiting their reabsorption by the kidney [Verzár & Laszt, 1934]. This phenomenon was explained by the further observation of the inhibiting effect of phloridzin, both on the phosphorylation of glycogen [Wilbrandt & Laszt, 1933] and on the glycolysis in muscle and yeast (also a phosphorylating mechanism [Lundsgaard, 1933]). As this inhibiting effect was also observed on glycolysis in brain, which is generally considered to be of a non-phosphorylating nature [Ashford & Holmes, 1929], there may be reason to suggest that phloridzin, besides inhibiting phosphorylation, may exert still another metabolic effect. Underhill [1912] postulated a twofold action: (1) on the permeability of kidney tissue and (2) "upon other structures, resulting in the production of sugar". It was the possibility of this second action of phloridzin on the production of sugar which presented the problem for this work. As biological material, liver of starved rats, in which carbohydrate synthesis has previously been demonstrated in experiments *in vitro* [Bach & Holmes, 1937] was chosen. Experiments on the effect of phloridzin and other glycolytic inhibitors on such carbohydrate production, which revealed a special behaviour of phloridzin, led to further investigations.

## *Methods*

*Animals.* Young rats were used, chiefly albinos, all males with an average weight of 200 g. They were always starved for 20–24 hr. before the experiments, in order to decrease the carbohydrate content of the liver. Before starving, the animals were kept on the ordinary stock laboratory diet.

*Slice technique.* This was the same as described previously [Bach and Holmes, 1937]. The weight of tissue was 100 mg. wet weight in all manometric experiments and varied from 300 to 500 mg. in experiments where the carbohydrate content was determined.

The slices were placed into Barcroft cups containing 3 ml.  $\text{NaHCO}_3$ -Ringer of pH 7.3 which included the substrates after adjustment to the same pH. The mixtures were equilibrated with a gas mixture containing 95 %  $\text{O}_2$  and 5 %  $\text{CO}_2$  in the aerobic, and with a mixture (passed over hot Cu filings) of 95 %  $\text{N}_2$  and 5 %  $\text{CO}_2$  in the anaerobic experiments.

In the experiments in which determinations of  $\text{O}_2$ ,  $\text{CO}_2$  and carbohydrate were carried out at different intervals, the time at which the vessels were placed in the water bath for incubation (before equilibration) was taken as zero.

*Manometric experiments.* The  $\text{O}_2$  uptake was determined in phosphate buffer, pH 7.3, with Barcroft manometers containing air and with 10 % KOH in the

centre pot of the vessels. For the determination of the R.Q. the method of Dixon & Keilin [1933] was employed, and was checked in one case by the Warburg two-vessel method. The latter experiment in which glycolysis, separately determined, was allowed for was carried out with the kind help of Dr H. Laser according to Laser & Rothschild [1939].

*Determination of total fermentable carbohydrate.* In most experiments the procedure previously described [Bach & Holmes, 1937] was closely followed and the final sugar estimations were performed by the method of Hagedorn & Jensen [1923]. In some of the "timing" experiments in absence of phloridzin (Figs. 3 and 5) the quicker, colorimetric method of Dische & Popper [1926] was used. Blank experiments showed that the two methods agreed within 5 %. For the experiments with phloridzin the Hagedorn & Jensen method was found to be the more reliable.

Inorganic phosphate was estimated according to the method of Fiske & Subbarow [1925] and lactic acid by the method of Friedemann *et al.* [1927].

*Reagents.* Phloretin was prepared in the following way: to 2 g. phloridzin suspended in 20 ml. boiling  $H_2O$ , 10 ml. 20 %  $H_2SO_4$  were added. The mixture was heated for 90 min. on the boiling water bath. The precipitate was filtered and thoroughly washed with cold water, dried and recrystallized from abs. alcohol. 90 % yield of carbohydrate-free phloretin was obtained. M.P. 253–255°.

The other reagents including phloridzin and insulin were supplied by British Drug Houses Ltd. The potency of the insulin hydrochloride was close to 20 units per mg.

#### EXPERIMENTAL

Liver slices were incubated in presence and absence of NaF, iodoacetate and phloridzin. The carbohydrate content of the tissue suspension was estimated before and after the period of incubation. Results from Table I show an increase

Table I. *The effect of phloridzin, NaF and iodoacetate on carbohydrate synthesis*

Quantities: mg. fermentable carbohydrate per g. fresh liver. Experimental period 2 hr.

			Extra increase with added		
Initial	Final (no addition)	Increase without addition	NaF 0.01 M	Iodoacetate 0.01 M	Phloridzin 0.01 M
2.93	5.77	+ 2.84	– 0.37	– 1.00	+ 2.26
—	5.23	—	– 0.60	– 0.51	—
2.92	4.39	+ 1.47	– 1.04	– 0.46	—
4.72	5.94	+ 1.22	—	—	+ 0.71
2.09	3.40	+ 1.31	—	—	+ 0.71
4.33	5.34	+ 1.01	—	—	+ 0.97*
7.30	7.50	+ 0.20	—	—	+ 1.40†

\* 0.02 M Phloridzin.

† 0.005 M Phloridzin.

in fermentable carbohydrate content in absence of substrates. This synthesis of carbohydrate was partly suppressed in presence of NaF and iodoacetate and was increased in presence of phloridzin. Blank experiments were carried out for all the substances added and special care was taken to allow for the possibility of the carbohydrate constituent of phloridzin being split off during the proceedings necessary for the determination of carbohydrate.

Since the substances added in these experiments are known to exert an inhibiting effect on a possible phosphorylating glycolysis, changes in the inorganic P content were expected during the period of incubation. Results in Table II however revealed a marked breakdown of organic P. The effect was of about the same order under both aerobic and anaerobic conditions and phloridzin

Table II. *Increase in inorganic phosphate content during incubation of liver slices*

Quantities: mg. inorganic phosphate per g. liver. Experimental period 2 hr. Concentration of added substances: phloridzin, 0.01 *M*; NaF, 0.01 *M*; glycogen, 2 %.

Substances added other than phloridzin	Increase in inorganic phosphate			Phloridzin effect
	Initial	Without phloridzin	With phloridzin	
Aerobic experiments				
—	0.47	+0.17	+0.14	-0.03
—	0.59	+0.27	+0.18	-0.09
Anaerobic experiments				
—	0.47	+0.19	+0.15	-0.04
—	0.59	+0.53	+0.40	-0.13
NaF	0.67	+0.35	+0.35	0
NaF + glycogen	0.67	+0.30	+0.26	-0.04

only slightly diminished this breakdown of organic P. NaF caused no significant effect, while the small phloridzin effect disappeared when NaF was added simultaneously. Finally glycogen was added to the NaF-poisoned tissue for the purpose of enriching it in glycolysing material. The phosphate breakdown was only slightly diminished by the presence of glycogen and a little further reduced when phloridzin was added in addition.

Table III shows results of lactic acid estimations carried out in a number of experiments before and after the incubation of liver slices. Here again no consistent nor significant changes in either direction in the lactic acid content were

Table III. *Changes in lactic acid content during incubation of liver slices*

Quantities: mg. lactic acid per g. liver. Experimental period 2 hr.

	Initial	Final	Change	Substances added
Aerobic	3.46	3.75	+0.29	—
„	3.70	3.62	-0.08	—
„	2.31	2.69	+0.38	—
„	3.41	3.20	-0.21	—
„	3.41	3.52	+0.11	Insulin 0.15 mg./ml.
Anaerobic	3.40	4.05	+0.65	—
„	3.40	3.76	+0.36	Phloridzin 0.01 <i>M</i>

observed when starved animals were used. A slight diminishing effect of phloridzin on glycolysis appeared under anaerobic conditions. Under aerobic conditions part of the lactic acid may have been oxidized, thus masking a possible formation of lactic acid. But the values obtained under anaerobic conditions were found to be of about the same order.

Finally experiments under anaerobic conditions, which eliminate both carbohydrate synthesis and carbohydrate oxidation, showed no significant changes in the carbohydrate content of the liver slices before and after the period of incubation (Table IV).

Summarizing the results of Tables II, III and IV there appears to be little evidence for the assumption that the increase in carbohydrate synthesis caused by phloridzin (Table I) may be due to an inhibition of the glycolytic disappearance of carbohydrate.

A possible effect of phloridzin on the oxidative disappearance of carbohydrate was investigated next. Figs. 1 and 2 show the rate of oxidation in liver tissue

Table IV. *Changes in carbohydrate content during incubation of liver slices under anaerobic conditions*

Quantities: mg. fermentable carbohydrate per g. liver. Experimental period 2 hr.

Initial	Final	Change	Substances added
10.86	10.00	-0.86	—
11.50	11.85	+0.35	—
4.61	4.70	+0.09	—
2.34	2.35	+0.01	Pyruvate 0.4 %
4.67	5.17	+0.50	Lactate 0.4 %
4.67	4.47	-0.20	Phloridzin 0.01 M

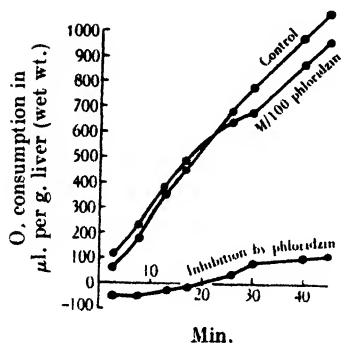


Fig. 1.

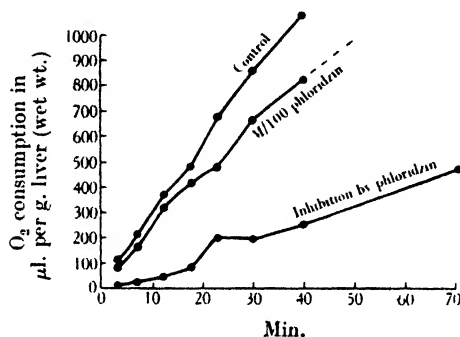


Fig. 2.

Figs. 1 and 2. The effect of phloridzin on the  $O_2$  consumption of liver slices. 0.1 g. tissue (wet wt.) in 3 ml. phosphate buffer, pH 7.3 in air at  $37^\circ$ .

from starved rats with and without added phloridzin. It will be seen that oxidation is slightly inhibited by phloridzin, the inhibition being greatest between 20 and 30 min. This phenomenon was observed in several successive cases. This inhibiting effect of phloridzin on the tissue oxidation was more understandable when the course of carbohydrate synthesis was followed by estimating the carbohydrate content of the slices at several intervals in the experimental period (Fig. 3). In all experiments a marked decrease of carbohydrate content was observed between 20 and 30 min., reaching values even below initial carbohydrate content. This phenomenon may be explained by the assumption that the rate of synthesis during the period mentioned was slower than that of carbohydrate consumption. And as, for reasons stated above, the possibility of a mainly glycolytic disappearance of carbohydrate can be disposed of, one may be justified in correlating the phenomenon observed in Figs. 1 and 2 with that of Fig. 3. It will be seen that the critical time of carbohydrate disappearance in absence of phloridzin is almost identical with that of the greatest inhibition of  $O_2$  consumption in presence of phloridzin. This may be a mere fortuitous coincidence, but one is tempted to connect the two results by arguing that phloridzin under the experimental conditions may inhibit an oxidative disappearance of carbohydrate in the critical period of 20–30 min. For, if the decrease in carbohydrate content was due to oxidation, and further if such oxidation was inhibited by phloridzin, a depression in  $O_2$  consumption in that critical period would be expected. The regularity of such depression of  $O_2$  uptake, as observed in Figs. 1 and 2, is shown in Table V in which the inhibiting effect of phloridzin on the  $O_2$  consumption during the period of 15–30 min. was calculated for three experiments, showing an average inhibition of  $120 \mu\text{l. } O_2$  per g. liver.

Table V. *O<sub>2</sub> consumption from 15 to 30 min. during incubation of liver slices with and without added phloridzin*Quantities:  $\mu$ l. O<sub>2</sub> per g. liver.

In absence of phloridzin			In presence of phloridzin			
After 15 min.	After 30 min.	During the interval of 15-30 min.	After 15 min.	After 30 min.	During the interval of 15-30 min.	Inhibition by phloridzin
352	773	421	375	683	308	113
497	862	365	412	667	255	110
530	883	353	310	526	216	137
Average inhibition between 15 and 30 min.						120

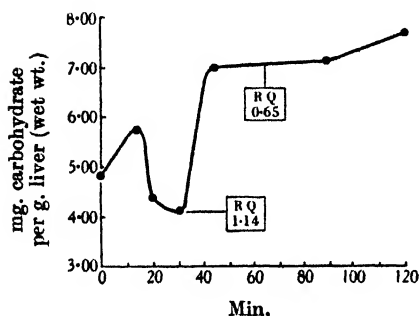


Fig. 3.

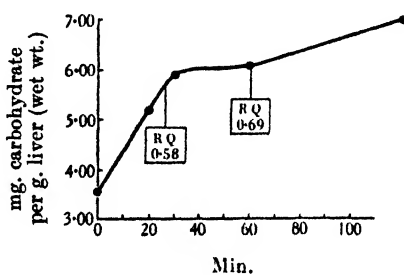


Fig. 4.

Fig. 3. The rate of carbohydrate synthesis in liver slices in absence of added substrate. 0.3-0.4 g. tissue in 3 ml. bicarbonate Ringer, pH 7.3 at 37°. No phloridzin.

Fig. 4. The effect of phloridzin on carbohydrate synthesis in liver slices. 0.5-0.35 g. tissue in 3 ml. phloridzin bicarbonate Ringer, pH 7.3 at 37°. Phloridzin concentration  $M/200$ .

Further evidence for this concept is given in Fig. 4, which shows the course of carbohydrate synthesis in presence of phloridzin. It will be seen that no decrease in carbohydrate content was observed during the critical period of 20-30 min. This was to be expected if phloridzin inhibited carbohydrate oxidation, thus preventing the carbohydrate content from decreasing in the period mentioned.

Experiments with insulin which according to previous findings partly inhibits the mechanism of synthesis of carbohydrate [Bach & Holmes, 1937] dispose of the idea that the disappearance of carbohydrate in the early period of incubation may be mainly due to an inhibition of carbohydrate synthesis in that period. Fig. 5 shows no significant effect of insulin on the O<sub>2</sub> consumption and from Fig. 6 it can be seen that the same decrease in carbohydrate content occurs at the critical time in presence of insulin, as has already been demonstrated in its absence (Fig. 3).

Finally determinations of the R.Q. carried out at two different periods of incubation in absence and presence of phloridzin supported the views stated above. If in the absence of phloridzin carbohydrate combustion was predominant in the early stage of incubation, the R.Q. for that period would be expected to approach 1. On the other hand a lower R.Q. should appear in presence of phloridzin, if carbohydrate combustion were partly suppressed. From Figs. 3 and 4 it can be seen that this is actually the case. The R.Q. in the critical time of the early stage of incubation was found to be 1.14 in absence of phloridzin and 0.58 in presence of phloridzin. For the later stage of incubation after 60 min., which

seems to be comparatively unaffected by phloridzin, the respective values were found to be 0.65 and 0.69. The latter phenomenon can be foreseen if carbohydrate oxidation takes place more rapidly in the early stage of incubation, an assumption supported by the results of Fig. 3, and further if, in accordance with the view stated above, it is this oxidation which is mainly affected by phloridzin.

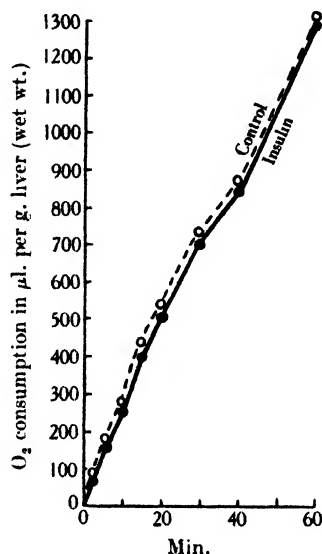


Fig. 5.

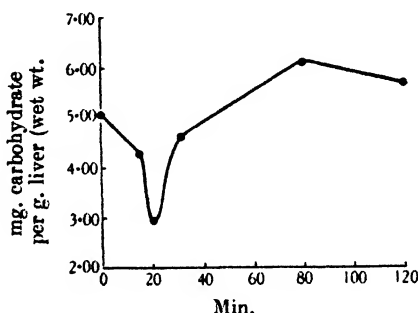


Fig. 6.

Fig. 5. The effect of insulin on the respiration of liver slices. 0.1 g. tissue in 3 ml. phosphate buffer, pH 7.3 in air at 37°.

Fig. 6. The rate of carbohydrate synthesis in liver slices in presence of insulin. 0.3–0.4 g. tissue in 3 ml. insulin bicarbonate Ringer, pH 7.3 at 37°. Insulin concentration 15 mg./100 ml.

Further it had to be considered whether phloridzin, being a glucoside, may be hydrolysed during incubation, thus increasing the carbohydrate content of the tissue suspension. This possibility could be excluded by two different types of experiments. Firstly slices were incubated in presence of phloridzin under anaerobic conditions, when both carbohydrate synthesis and carbohydrate oxidation would be eliminated, whereas a possible hydrolysis of phloridzin could still take place. As shown in Table IV, however, the carbohydrate content remained practically unchanged, indicating absence of hydrolysis. Secondly, phloretin was prepared from phloridzin by acid hydrolysis as described earlier. Care was taken to remove the glucose component completely, 97 % of the theoretical amount of which was determined in the hydrolysate. Unlike phloridzin the  $O_2$  consumption of liver slices was inhibited by 52 % at a concentration of 0.01  $M$  of the phloretin suspension, the comparative insolubility of the substance serving probably as limiting factor for its inhibitory effect. This was shown by the fact that inhibition was the same in all concentrations down to 0.002  $M$  (Fig. 7). At 0.001  $M$  the inhibition of  $O_2$  consumption was still 30 %, whereas carbohydrate synthesis was hardly at all affected at that concentration, as compared with 49 % inhibition of synthesis at 0.01  $M$  (Table VI). In an attempt to imitate conditions prevailing in the case of phloridzin, glucose was added to the phloretin-Ringer



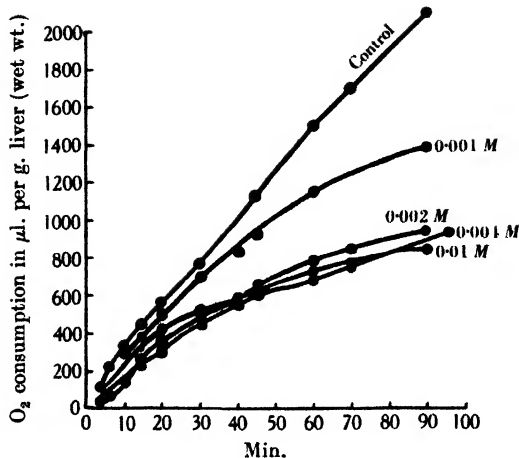


Fig. 7. The effect of phloretin on the respiration of liver slices. 0.1 g. tissue in 3 ml. of phloretin phosphate buffer, pH 7.3 in air at 37°.

Table VI. *The effect of phloretin on O<sub>2</sub> consumption and carbohydrate synthesis in liver slices*

Experimental period 2 hr.

Phloretin concentration <i>M</i>	O <sub>2</sub> consumption in µl. per hr. and g. liver (wet wt.)			Carbohydrate synthesis (mg. fermentable carbohydrate per g. liver)		
	Without phloretin	With phloretin	Inhibition %	Without phloretin	With phloretin	Inhibition %
0.01	1505	730	52	2.33	1.20	49
0.004	1590	745	53	—	—	—
0.002	1580	790	50	—	—	—
0.001	1620	1140	29.5	2.34	2.19	6

Table VII. *The effect of glucose in presence of phloretin on carbohydrate synthesis in liver slices*

Quantities: mg. fermentable carbohydrate per g. liver. Experimental period 2 hr.

Substances added	Before incubation	After incubation	Increase	Glucose effect in presence of phloretin
None	1.60	3.93	2.33	+ 0.46
Glucose 0.2 %	6.25	8.04	1.79	
Phloretin 0.001 <i>M</i>	1.60	3.29	1.69	
Phloretin and glucose	6.51	8.66	2.15	
None	3.20	5.53	2.33	+ 1.26
Phloretin 0.001 <i>M</i>	3.20	5.12	1.92	
Phloretin and glucose	6.96	10.14	3.18	

(0.001 *M*). It will be seen from Table VII that phloretin in presence of added glucose increased the total carbohydrate content of the liver slices by an amount exceeding the carbohydrate synthesis shown by the controls. This seems to indicate that the phloridzin effect is due to the complete glucoside rather than to either of its constituents. This experiment also confirms the view that the increase of carbohydrate content resulting from incubation of liver slices with phloridzin is not due to the liberation of the glucose component of the glucoside.

## DISCUSSION

The impairing effect of phloridzin on the mechanism concerned with the removal of carbohydrate from the blood has been recognized for a long time [Goldstein *et al.* 1932]. The discovery of its inhibitory effect on phosphorylating carbohydrate fermentation as described earlier stimulated research in that particular direction. But when the carbohydrate-sparing effect of phloridzin was observed in tissues which either, as in the case of liver, show little glycolytic activity or, as in the case of brain, are known to catalyse non-phosphorylating glycolysis [Ashford & Holmes, 1929], other possibilities had to be considered. In the particular case of this work the increased synthesis of fermentable carbohydrate in liver slices observed in presence of phloridzin was interpreted, in agreement with the views stated earlier, as an inhibited removal of carbohydrate. The observation of only small changes in inorganic phosphate content in presence and absence of glycolytic inhibitors may not alone be sufficient evidence against the assumption of a glycolytic mechanism, possibly inhibited by phloridzin, although the absence of such a mechanism has been reported by several other workers [Rona *et al.* 1925; Dann & Quastel, 1928; Tanko, 1931]. On the other hand the considerable breakdown of organic phosphate found in accordance with Rona *et al.* [1925] and Toerell & Norberg [1932] may have masked any possible rephosphorylation. But the fact that no lactic acid formation was found to take place during the incubation of liver slices (in accordance with Haarmann & Brink [1935] and Willstätter [1936]) and further, the fact that the carbohydrate level remained unchanged under anaerobic conditions, supplies additional evidence that the disappearance of carbohydrate observed in absence and inhibited in presence of phloridzin could not be of fermentative nature.

Now it is known from experiments *in vivo* that though hepatic glycogen is greatly diminished following phloridzin administration, the glucæmic level remains normal or even shows a marked increase [Schwarz & Sassler, 1928; Rathery *et al.* 1930]. The two phenomena may be the result of two separate mechanisms but the accumulation of glucose, i.e. of a readily oxidizable substance in presence of phloridzin, suggests a disturbance of the oxidative function of the liver [Nash & Benedict, 1923]. The experiments *in vitro* on the isolated organ described in this work may supply satisfactory confirmation of this view, suggested hitherto by experiments on the whole animal. The period of inhibition of  $O_2$  consumption by phloridzin coincides well with the period of carbohydrate disappearance in its absence, thus explaining the carbohydrate-saving effect. Fleischmann [1937] observed a similar type of  $O_2$  inhibition with phloridzin in brain slices, but failed to do so in liver or kidney slices. This result may have technical causes since he also failed to observe the familiar extra  $O_2$  uptake after adding glucose to these organs.

Estimations of the respiratory quotient fitted in well with the views stated above, as it could be shown that in presence of phloridzin the R.Q. was lowered at the same period of incubation in which both carbohydrate disappearance and respiration were inhibited. Here again the lowering of the R.Q. by phloridzin falls in line with similar observations made *in vivo* [Bonsignore & Cavaglione, 1936]. These workers, using guinea-pigs, observed no increase, when glucose was administered, of the R.Q. lowered by phloridzin poisoning.

The almost toxic behaviour of phloretin in liver slices of starved rats is particularly interesting as it was possible to reverse its inhibitory effect on carbohydrate synthesis by adding glucose, in fact to make it behave like phloridzin. It seems feasible to assume that phloretin administered to the body may

have carbohydrate at its disposal, and may therefore be able to produce physiological effects similar to those of phloridzin. This is in accordance with the findings of Lambrechts [1936] who observed glucosuria following the administration of phloretin to chloralosed dogs.

#### SUMMARY

1. Carbohydrate synthesis in liver slices is partly suppressed by NaF and iodoacetate but is apparently increased in presence of phloridzin. The increase was shown to be the result of an inhibition of carbohydrate disappearance by phloridzin in the early stage of incubation. Evidence was produced that this disappearance, which was unaffected by insulin, was due to oxidation rather than to fermentation of the carbohydrate. Hence it was concluded that phloridzin inhibits carbohydrate oxidation *in vitro*.

2. The above view was supported by a regularly occurring inhibition of  $O_2$  consumption by phloridzin at the same period of incubation at which in its absence carbohydrate oxidation mainly takes place. Further evidence for this view was given by the fact that the R.Q. for the above period approached unity in absence and was found to be 0.58 in presence of phloridzin.

3. Phloretin, unlike phloridzin, partly inhibits both  $O_2$  uptake and carbohydrate synthesis in liver slices in concentration higher than 0.001 *M*. But when glucose was added simultaneously, phloretin showed an effect similar to that of phloridzin on carbohydrate synthesis. This indicates that the phloridzin effect is probably due to the complete glucoside rather than to its constituents.

4. The above phenomenon and further the fact that the carbohydrate level of liver tissue remained unchanged when incubated with phloridzin under anaerobic conditions dispose of the idea of a possible hydrolysis of the glucoside during the period of incubation, which otherwise could have vitiated the observations.

I wish to express my thanks to Sir F. G. Hopkins for his interest in this work.

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## CII. THE MECHANISM OF THE BIOLOGICAL SYNTHESIS OF ACETYLCHOLINE. II

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*(Received 7 March 1939)*

IN our first publication [Stedman & Stedman, 1937] on this subject it was shown that the addition of sodium acetoacetate to minced brain tissue, treated with chloroform under specified conditions, caused an appreciable increase in the formation of acetylcholine in such tissue when it was incubated at 37°. Subsequent work [Stedman & Stedman, 1938] has confirmed this result, but publication of the details of our experiments has hitherto been withheld owing to the interpretation which Mann *et al.* [1938, 1, 2] have placed upon our findings, an interpretation which has necessitated an investigation of certain aspects of our work which is more detailed than that previously carried out.

### *Experimental methods*

Ox brain has, in general, been used in this work. The brains were brought from the slaughter-house to the laboratory as quickly as possible after the slaughter of the animal. Since, under the conditions of our experiments, the formation of acetylcholine is greater in the basal ganglia than in the remainder of the brain, this material has, as far as possible, been employed. Unfortunately, however, the corpora striata, in which the formation of acetylcholine is, of the material examined, greatest, were frequently damaged or completely destroyed during the slaughter of the animal. It has therefore been necessary to use mainly a mixture of the thalami with cortical tissue from the hemispheres. The tissue so selected was first finely minced and then thoroughly ground in a mortar. 10 g. portions of the material, thus rendered as uniform as possible, were then used in the subsequent experiment. In general, each portion was first separately ground in a mortar with 5 ml. of an organic solvent containing 5 mg. of eserine base, after which additions of further substances dissolved in a small volume of saline or phosphate Ringer (pH 7.4) were, if necessary, made. Where required, suitable controls were prepared at the same time. After an experimental period of 2 hr. at the required temperature the material was quickly and thoroughly mixed with 50 ml. of absolute alcohol and left overnight. For the preparation of an extract, suitable for assay or comparison, of the acetylcholine present, the alcohol was filtered and the residue twice thoroughly washed with more of the solvent. The combined filtrate and washings were evaporated to dryness under diminished pressure at low temperature, and if, as often happened, the material could not be submitted immediately to the remaining stages of purification, the dry residue was stored in an evacuated desiccator over  $\text{H}_2\text{SO}_4$ . In any case, it was next intimately mixed with a total volume, including that used for washing purposes, of 20 ml. of 10% trichloroacetic acid, centrifuged to remove insoluble material, and the centrifugate extracted 4 times with ether to remove the bulk of the trichloroacetic acid. The solution was now made just alkaline to litmus by the addition of  $\text{NaHCO}_3$ , extracted 5 times with chloroform to remove the eserine, and, after again making acid with tartaric acid, evaporated to dryness under diminished pressure at low temperature. The dry residue was stored, as

before, in a desiccator. This process of purification, although long and tedious, has been used throughout this work because it not only removed a large amount of impurity from the alcoholic extracts but also yielded a product which was completely and readily soluble in water. The assay or comparison of the amount of acetylcholine in these extracts has usually been made on the dorsal muscle of the leech. Occasionally, however, strips of the small intestine of the rabbit or of the frog's auricle have been used as test objects.

#### *Efficacy of method of extraction*

Apart from the probability of its greater efficiency, the choice of alcohol rather than of aqueous solvents for the extraction of acetylcholine from brain tissue was made largely because it was thought that dilution of the tissue with a relatively large volume of a dehydrating solvent would rapidly arrest any enzymic processes which might be involved in the production of acetylcholine. Its use possessed, moreover, the additional practical advantage that the alcohol could be much more readily filtered from the residual solid than could, for example, aqueous trichloroacetic acid. It became necessary, however, to test the relative efficacies of various solvents, particularly in view of the opinions which have been expressed regarding the occurrence in nervous tissue of a bound form of acetylcholine. Such a possibility was discussed about 6 years ago by Chang & Gaddum [1933]. Since that time Corteggiani *et al.* [1936; 1937] have stated that by heating brain tissue with physiological saline in the presence of eserine at 70° for 3 min. more acetylcholine passes into the solvent than when the extraction is carried out at room temperature, and they have attributed this result to the presence in the tissue of a complex which liberates acetylcholine under the influence of heat. Loewi *et al.* [1937; 1938] have similarly found that eserinated Ringer extracts considerably less acetylcholine from the central nervous system of the frog than does *N*/100 alcoholic HCl, and have also concluded that a combined form of acetylcholine is present in nervous tissue. More recently, Mann *et al.* [1938, 1, 2] have attributed the power of chloroform, described in our first communication, of producing a large increase in the amount of extractable acetylcholine present in brain tissue to the ability of this solvent to liberate acetylcholine from a complex in which it already exists in a preformed but combined condition. The properties ascribed by the last-mentioned authors to their hypothetical complex are of such a nature that it is impossible directly to disprove its existence. It can, however, be readily shown that such a complex, if it does exist, is quite different from that of Loewi. Thus, 10 g. of brain tissue were thoroughly ground with chloroform-eserine and kept for 2 hr. at room temperature, when 100 ml. of *N*/100 alcoholic HCl were added. Three similar portions of the same material were treated immediately with 100 ml. of alcohol, *N*/100 alcoholic HCl or 10% trichloroacetic acid respectively. Alcohol and alcoholic HCl extracted identical amounts of acetylcholine from the untreated tissue; trichloroacetic acid extracted slightly less, due probably to loss on account of difficulties in manipulation; while the tissue treated with chloroform yielded 9-10 times as much as the controls. According to Loewi, *N*/100 alcoholic HCl extracts both free and bound acetylcholine from nervous tissue. It follows that the increased yield of acetylcholine obtained after treatment with chloroform does not originate from the bound form of this substance which Loewi has demonstrated to exist in nervous tissue. The experiment shows, moreover, that alcohol, which we have consistently employed for the extraction of acetylcholine from tissue, is as efficient a solvent for this purpose as *N*/100 alcoholic HCl and is at least as efficient as 10% trichloroacetic acid. Experiments quoted

below in another connexion will also demonstrate that the amount of acetylcholine extracted by acetone is identical with that obtained with alcohol. The virtual identity of the yields obtained with these various solvents suggests that they all effect a complete extraction of acetylcholine, "bound" or free, from the tissue. This result does not appear to us to be in conformity with the view that the much larger amount of acetylcholine produced relatively slowly in the presence of chloroform is already preformed in the tissue.

*Influence of temperature on the chloroform effect*

When we first demonstrated the power of chloroform to increase the formation of acetylcholine in brain tissue, our experimental procedure involved the incubation of the material at 37°. No reason was given for the choice of this particular temperature, and, beyond the unverified assumption that any effect which chloroform might possess would be greater at body than at other temperatures, none, in fact, existed. Mann *et al.* [1938, 1] have, however, pointed out that the effect of chloroform is greater at room temperature than at 37°. They claim, moreover, that it is as great at 0° as at 37°. It must be confessed that if this were the case and if amounts of acetylcholine comparable with those formed at higher temperature were, in fact, liberated at 0°, the argument that such acetylcholine represented newly synthesized material would be considerably weakened. Many experiments, of which the following is typical, have therefore been carried out to test this point.

Four portions of brain tissue were separately treated with chloroform- eserine. Of these, the control was mixed immediately with alcohol, while the remainder were kept for 2 hr. at 0°, room temperature and 37° respectively. The acetylcholine present was then extracted and the yields compared on the leech muscle. The results showed that the ratios of the amounts of acetylcholine obtained were: control: 0°: 37°: room temperature = 1: 1.4: 5: 6.5, from which it follows, after deducting the value of the control from the remainder, that the amounts of acetylcholine produced during the experiment were in the ratio 0°: 37°: room temperature = 1: 10: 13.8. It is thus clear that the amount of acetylcholine produced at 0° is only 1/14 and 1/10 of that formed at room temperature and 37° respectively. This amount, while not entirely negligible, is so small that it appears justifiable to assume that it is mainly produced during the short period necessary to reduce the temperature of the material to 0°. Similar results were obtained in other experiments, in some of which the acetylcholine was assayed on strips of rabbit's intestine.

Chloroform evidently exerts a dual action on brain tissue. In addition to facilitating the formation of acetylcholine, it also effects a destruction of the mechanism responsible for this formation. At 37° the latter process is much accelerated; hence the somewhat smaller yield of acetylcholine. That this is an adequate explanation of the phenomenon appears to be confirmed by the facts, which will be demonstrated below, that ether is superior to chloroform in its ability to cause the formation of acetylcholine in brain tissue, and that its effect is, moreover, greater at 37° than at room temperature. The destructive action of ether on the mechanism involved is apparently either smaller than that of chloroform or is entirely absent.

*Effect of various organic solvents on the formation of acetylcholine*

In attempting to elucidate the mechanism of the action of chloroform in increasing the amount of extractable acetylcholine in brain tissue, we have carried out a number of experiments in which chloroform has been replaced

by other solvents. These quickly demonstrated that ether is more effective both at room temperature and at 37° than chloroform under the same conditions. Experiments with acetone at room temperature, however, gave yields of acetylcholine identical with those obtained both in the usual control and in one in which the 10 g. sample of brain tissue was treated immediately with 100 ml. acetone. Alcohol, on the other hand, yielded, at room temperature, slightly more acetylcholine than the control. In two experiments, again at room temperature, a quantitative comparison was made of the yields obtained with the effective solvents. In one of these, six times as much acetylcholine was obtained from the sample treated with ether, and 3.2 times as much from that treated with chloroform, as from the one treated with alcohol. In the second the usual control was also made. The results can be summarized by the ratio control : alcohol : chloroform : ether = 1 : 1.9 : 4.1 : 7. If the amount formed in the control is deducted from the remainder, the ratio alcohol : chloroform : ether = 1 : 3.5 : 6.7 is obtained. It is apparent from these results that the two strongly dehydrating solvents, alcohol and acetone, both destroy the mechanism responsible for the formation of acetylcholine in brain tissue. Acetone is, however, evidently the more effective in this respect since some formation continues in the presence of alcohol unless relatively large volumes, such as we use in the extraction of the acetylcholine, are employed. The fact that the yield obtained from brain tissue after treatment with chloroform is less than that produced in the presence of ether can thus clearly be attributed to a similar, but less rapid, destruction of the mechanism in question by this solvent. Not only, however, does ether cause a greater formation of acetylcholine at room temperature than chloroform, but it also differs from the latter solvent in that its effect is increased at 37° whereas that of chloroform is diminished. This is apparent from Fig. 1.

*Effect of various substances on the formation of acetylcholine*

We have already stated [Stedman & Stedman, 1938] that the addition of sodium acetoacetate to brain tissue treated with chloroform-escrine caused an increase of 100 % in the yield of acetylcholine extractable from such tissue, and that the addition of choline produced a further, but smaller, increase in the yield. In the experiments, which have not hitherto been recorded, on which this statement was based, the brain tissue was prepared according to our standard procedure, four portions being separately treated with chloroform-escrine and then ground with 2 ml. of saline without further addition or with the same volume of saline containing 10 mg. of choline chloride, 20 mg. of sodium acetoacetate or of both these substances respectively. The material was incubated for 2 hr. at 37° when the acetylcholine was extracted, acetoacetate and/or choline in the above amounts being added to the alcoholic extracts of the samples which had not been treated with these substances, and a comparison made of the amounts obtained from the various portions. Some of the leech tracings are reproduced in Fig. 2. An examination of the responses shows that, both in the presence and absence of acetoacetate, choline causes a small but definite increase in the yield of acetylcholine. A similar and subsequent experiment, moreover, completely confirmed this result. Nevertheless, it must be recorded that in several experiments, identical with the above except that the material has been kept at room temperature instead of at 37°, we have since been unable to demonstrate any effect due to choline.

As regards the influence of acetoacetate, it is evident from Fig. 2 that this substance has approximately doubled the yield of acetylcholine. This rough

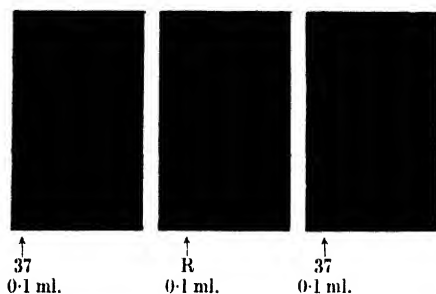


Fig. 1.

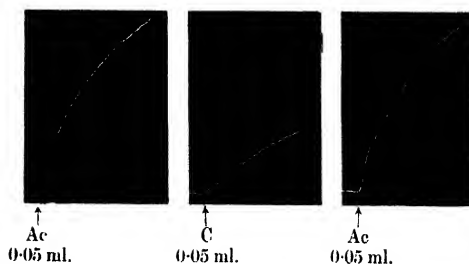


Fig. 3.

Fig. 1. Effect of temperature on the formation of acetylcholine in the ether preparation. R = kept at room temperature for 2 hr.; 37 = incubated at 37° for 2 hr. Final volume of extracts = 250 ml.

Fig. 3. Effect of acetoacetate on the formation of acetylcholine in the chloroform preparation at room temperature. Final volume of extracts = 50 ml. Ac = with added acetoacetate; C = control.

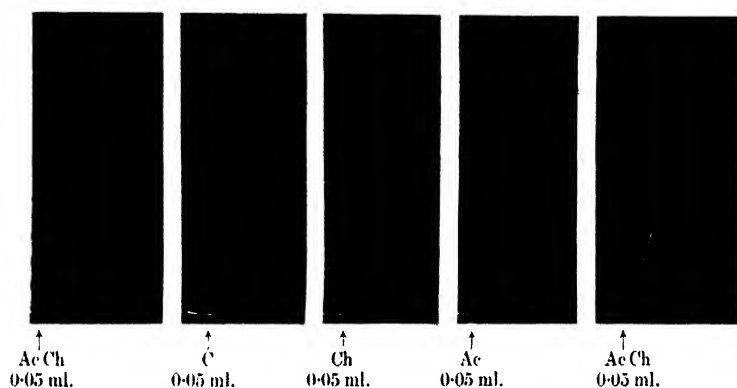


Fig. 2. Effect of sodium acetoacetate and of choline on the formation of acetylcholine in the chloroform preparation at 37°. For details, see text. Final volume of extracts = 250 ml. C = control; Ac = with added acetoacetate; Ch = with added choline; AcCh = with added acetoacetate and choline.

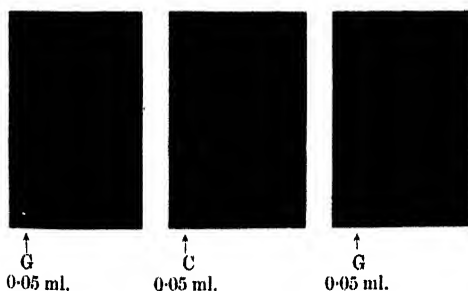


Fig. 4.

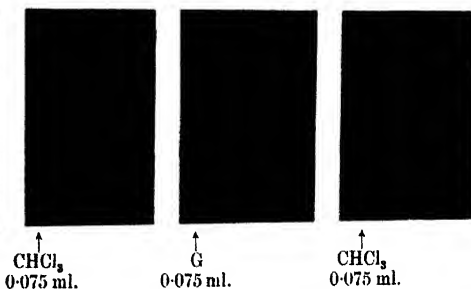


Fig. 5.

Fig. 4. Effect of glucose on the formation of acetylcholine by brain tissue suspended in phosphate-Ringer at 37°. Final volume of extracts = 15 ml. G = with added glucose; C = control.

Fig. 5. Continuation of Fig. 4, showing absence of effect on shaking with chloroform after incubation.  $\text{CHCl}_3$  = shaken with chloroform.



estimate of its effect has, moreover, been confirmed in numerous similar experiments in which more accurate comparisons with the controls have been made. Mann *et al.* [1938, 1] appear also to have confirmed this result, but they state that, even in the presence of acetoacetate, the amount of acetylcholine produced at 37° is no greater than that at room temperature in its absence. They further claim that acetoacetate does not increase the yield of acetylcholine if the tissue is kept at 18° in the presence of chloroform. This is entirely contrary to our experience. Fig. 3, for example, shows that addition of acetoacetate to the chloroform preparation at room temperature greatly increases the yield of acetylcholine, and similar results have been obtained on numerous occasions. Since there is thus no doubt that acetoacetate does increase the formation of acetylcholine in such preparations, the point as to whether or no the yield at 37° in the presence of acetoacetate is no greater than at 18° in its absence becomes immaterial.

We have previously shown [Stedman & Stedman, 1937] that addition of sodium pyruvate to brain tissue treated with chloroform- eserine diminishes the formation of acetylcholine in such tissue when it is incubated at 37°. In view of the above results, the effect of this substance has been examined at room temperature. It has been found that sodium pyruvate still exerts a considerable inhibitory action on the formation of acetylcholine under these conditions.

#### *Quantitative assays of the yield of acetylcholine*

In the preceding experiments the influence of any particular treatment on the formation of acetylcholine in brain tissue has been ascertained by directly comparing the response of the leech muscle to extracts of the treated tissue with similar extracts of suitable controls. While this is probably the most rapid, and certainly the most accurate method of determining the relative effects of the treatment, it nevertheless seemed desirable to measure the absolute amounts of acetylcholine produced under certain conditions. Some results of such measurements are recorded in the following experiments.

Of five 10 g. portions of minced and ground ox brain, one (control) was treated immediately with 50 ml. alcohol, two were ground with chloroform- eserine and two with ether- eserine, 1 ml. of saline or of a solution of sodium acetoacetate (20 mg.) in saline being added to the chloroform and ether preparations. After 2 hr. at room temperature alcohol was added to the latter preparations and the acetylcholine extracted in the usual manner. The results of the assays were: control, 1.3–1.4; chloroform, 6.0; ether, 8.7; chloroform + acetoacetate, 12.5; ether + acetoacetate, 14.5  $\mu$ g. per g. tissue.

Another specimen of brain tissue was treated with ether- eserine and 20 mg. sodium acetoacetate and incubated for 2 hr. at 37°. Assay showed the presence of 23  $\mu$ g. acetylcholine per g. extract. The control, treated in the same way except that no acetoacetate was added, gave a yield of about 17  $\mu$ g. per g.

We have not made extensive examination of nervous tissue from species other than the ox, but it seemed desirable to ascertain if the above values were peculiar to brain tissue from this animal or if values of the same order of magnitude were given by other species. We therefore carried out some preliminary experiments with the rat, this species being selected mainly because the brain could be obtained immediately on removal from the animal. One whole brain was minced and ground in a mortar without any addition. It was then treated with 50 ml. acetone and the extract worked up in the usual manner. Assay showed a content of 0.6  $\mu$ g. acetylcholine per g. tissue. Another whole brain was similarly prepared, ground with ether- eserine and incubated at 37° for 2 hr.

The yield of acetylcholine in this case was 16  $\mu$ g. per g. The numerical results from these two experiments, although obtained from different brains, differ so widely in magnitude that they leave little doubt that brain tissue from the rat is quite comparable with that from the ox as regards its production of acetylcholine under the influence of ether.

*Formation of acetylcholine in aqueous media*

It has previously been shown [Stedman & Stedman, 1937] that when brain tissue is ground with water containing eserine sulphate and the material incubated at 37° little, if any, acetylcholine is formed. Apparently water, like certain organic solvents, destroys the mechanism responsible for the production of acetylcholine in brain tissue. Nevertheless, Mann *et al.* [1938, 2] have found that when a suspension of brain tissue in eserinated phosphate Ringer is shaken at 37° in an O<sub>2</sub> atmosphere, there occurs an accumulation of acetylcholine in the suspension fluid. It thus seemed desirable to compare the amounts of acetylcholine formed under the influence of chloroform with those produced in aqueous media. For the latter experiments, 10 g. portions of brain tissue were ground with a total of 10 ml. phosphate Ringer (pH 7.4) or of physiological saline containing eserine sulphate and the suspension shaken in air under various conditions, 100 ml. alcohol being added at the termination of the experiment, after which the acetylcholine was extracted and assayed in the usual manner. The results show that while there is some formation of acetylcholine at room temperature, the amount is small compared with that produced at 37°. At the latter temperature, using a period of 2 hr., the amount produced is generally slightly smaller, but never greater, than that formed under the influence of chloroform at room temperature. If 5 ml. chloroform are added to the suspension in Ringer at the commencement of the experiment, the formation of acetylcholine is inhibited to a considerable degree at 37°. At room temperature, however, it is increased to a value approximately equal to that formed at the same temperature in the presence of chloroform alone. Without discussing these results in detail, it will, we think, be agreed that they can be best interpreted on the assumption that the acetylcholine formed in aqueous media has the same origin as that produced under the influence of chloroform. At room temperature, chloroform is much more efficient than Ringer: at 37°, however, its deleterious action again becomes apparent.

Many experiments have also been carried out with suspensions of brain tissue in phosphate Ringer at 37° to which 20 mg. glucose or of sodium pyruvate have been added. According to Mann *et al.* [1938, 2] the addition of these substrates should considerably enhance the yield of acetylcholine. In some experiments we did, indeed, observe a small but scarcely measurable increase, but in others none was detectable. We were inclined to attribute the discrepancy between our results and those of Mann *et al.* to differences in technique. Our preparations were, for example, shaken in air instead of in O<sub>2</sub>. The ox brain tissue which we employed was, moreover, necessarily less fresh than the rat brain tissue used by these authors. We therefore carried out some experiments with rat brains, of which the following are typical.

Four rat brains were finely minced and thoroughly ground in a mortar. Three 1 g. portions of the tissue were separately ground with 0.5 ml. saline containing 0.5 mg. eserine sulphate and washed into flasks with 5 ml. phosphate Ringer (pH 7.4). To one flask was added 1 ml. saline and to the remainder 1 ml. saline containing 10 mg. glucose. The flasks were then filled with O<sub>2</sub>, placed in a bath at 37°, and shaken for 2 hr. After cooling to room temperature, 0.5 ml.

chloroform was added to one of the flasks containing glucose and the contents vigorously shaken. All were then left for 1 hr. at room temperature, when the experiment was stopped by the addition of 50 ml. acetone to each flask. The acetylcholine was then extracted and assayed in the usual manner. Fig. 4 shows the effect on the leech muscle of equal volumes of extracts from the control (no added glucose) and from the portion to which glucose was added. It is clear from this that, while the yield of acetylcholine from the latter was slightly greater than from the control, the difference is so small as to be of doubtful significance. The same tracing is continued in Fig. 5, from which it is apparent that shaking with chloroform under the conditions used in this experiment causes no increase in the yield of acetylcholine.

Another experiment was carried out which was identical with the above except that the amount of glucose added was restricted to 2 mg. and the treatment of one portion with chloroform omitted. In this experiment a quantitative assay of the yield of acetylcholine was made. This showed that the quantity produced in the experiment with added glucose, which was identical within the limits of experimental error with that produced in its absence, was at least 18  $\mu$ g. per g.

It should be noted that the above results are in complete contradiction with those of Mann *et al.* [1938, 2] who claim (1) that the addition of glucose to the suspension medium causes a large increase in the yield of acetylcholine, and (2) that treatment with chloroform, as carried out in our experiment, causes a further increase. We are at a loss to explain the discrepancy between the two investigations. Our experiments were carried out under conditions as similar as possible to those of Mann *et al.* They differed merely in the facts that we (1) probably used rather more brain tissue, and (2) extracted the acetylcholine from both the suspension medium and the tissue with acetone instead of assaying that in the suspension medium alone. This method of extraction should not, however, remove acetylcholine from the hypothetical "preformed-precursor", for, as we have shown in our preceding experiments that formed as a result of treatment with chloroform, which is supposed to originate from the "preformed-precursor", is not extractable with acetone until such treatment has been carried out. We should nevertheless feel inclined to attribute our failure to demonstrate any effect of glucose to a difference in technique, were it not for the fact that the yield of acetylcholine which we obtained in the absence of added glucose is of the same order of magnitude as that obtained by Mann *et al.* in its presence. It is probable that the relative amount of suspension fluid which we employed was somewhat smaller than that used by Mann *et al.*, but we cannot think that the discrepancy is attributable to this.

## DISCUSSION

The ultimate aim of this investigation is to elucidate the mechanism of the biological formation of acetylcholine. Many of the experiments described above have, however, been carried out with the object of examining certain objections which Mann *et al.* have raised to our view that the acetylcholine formed in brain tissue when such tissue is incubated with chloroform is actually synthesized *in vitro*. Of these objections, the only one for which experimental foundation is claimed is that the yield of acetylcholine from a chloroform preparation is greater at room temperature than at 37°, and that it is, moreover, no greater at the latter temperature than at 0°. As we have shown above, only negligible amounts are, in fact, formed at 0°, but we agree that somewhat more is produced

at room temperature than at 37°. While there is no difficulty in supposing that the mechanism, present in nervous tissue, which is responsible for the synthesis of acetylcholine can function at temperatures of about 18°, as indeed it must so function in cold-blooded animals, it must be admitted that one would expect its efficiency to be greater at the higher temperature of 37°. This minor anomaly has, however, been completely explained by our experiments with ether. It can, we think, scarcely be disputed that the acetylcholine formed in the ether preparation originates from the same source, and by the same mechanism, as that produced in the chloroform preparation. But, as we have shown above, the amount of acetylcholine so formed in the ether preparation is not only greater than that in the corresponding chloroform preparation both at room temperature and at 37°, but the process exhibits a considerably greater efficiency at the higher temperature. It is, we think, clear from this result that the chloroform exerts a deleterious action on the mechanism of the process in question which is much more rapid at 37° than at room temperature.

This explanation of the anomalous temperature effect in the action of chloroform does not, however, entirely dispose of the claim of Mann *et al.* that there exists in brain tissue a "preformed-precursor" of acetylcholine. It is therefore necessary to consider this claim in more detail, for it must be admitted that if the ability of chloroform and, presumably, ether to increase the amount of extractable acetylcholine in brain tissue is merely due, as these authors suggest, to their power of releasing such acetylcholine from a precursor in which it exists in a preformed condition, then experiments such as we have carried out must necessarily be incapable of providing information regarding the mechanism of the biological formation of acetylcholine.

What, then, is the evidence for the existence of the "preformed-precursor" of Mann *et al.*? Briefly, their claim is based largely on the following observations: (1) When a suspension of brain tissue in certain aqueous media is shaken at 37° in O<sub>2</sub>, there occurs an increase in the acetylcholine content of the suspension medium. (2) If, after such increase has occurred, the suspension is shaken with chloroform or brought to pH 3.0 and left at room temperature for a period of from 30 to 90 min., a further increase takes place. The second increase is supposed to demonstrate the existence, and to be a measure of the amount, of the "preformed-precursor" in the tissue after incubation. Now, it is to be noted that Mann *et al.* only measure the acetylcholine content of the suspension medium. According to Loewi, the aqueous media which they employ are incapable of completely extracting this substance from nervous tissue, and one is therefore justified in concluding that there exists a residue of unextracted acetylcholine in their material. Loewi has further shown that aqueous acids, as well as alcoholic HCl, liberate this "bound" acetylcholine. It is therefore not surprising that the yield of acetylcholine in the suspension fluid slowly increases after acidification. So far the "preformed-precursor" of Mann *et al.* appears to be nothing else than the "bound" acetylcholine of Loewi. But Mann *et al.*, utilizing under somewhat different conditions our observation that treatment of brain tissue with chloroform causes a large increase in its content of acetylcholine, claim to have demonstrated by this means the existence of their "preformed-precursor" and simultaneously to have proved that the acetylcholine produced in our experiments had not been synthesized *in vitro* but had pre-existed in the tissue. While it has not been definitely proved, it must be presumed, unless evidence to the contrary is provided, that the additional acetylcholine formed under the influence of chloroform in the experiments of Mann *et al.* has the same origin as that produced by the same agent under the

conditions which we employ. If, however, this presumption be accepted, it is clear that the source of this acetylcholine cannot be the "bound" acetylcholine of Loewi, for our experiments have proved without doubt that treatment of brain tissue with chloroform causes the formation in such tissue of an amount of acetylcholine many times greater than can be extracted from similar but untreated tissue by solvents which remove Loewi's "bound" acetylcholine, and we are thus faced with the prospect of dealing with the presence of two different bound forms of the substance. Actually, however, there is at present no evidence for the existence of the "preformed-precursor" as distinct from Loewi's "bound" form. Its conception is merely an interpretation of our discovery of the influence of chloroform. Mann *et al.*, in fact, virtually argue that its presence is proved by the ability of chloroform to cause the formation of acetylcholine in brain tissue, and hence that such acetylcholine must originate from it.

The quantitative data which we have provided give, moreover, no evidence in support of the existence of the "preformed-precursor". Thus, minced and ground ox brain which we use in our experiments contains a total, including Loewi's "bound" acetylcholine, of about 1.5  $\mu$ g. acetylcholine per g. Similar tissue from a rat's brain contains even less. Yet by incubating such tissue with ether, the effect of which must clearly be similar to that of chloroform, these amounts can be raised to 17 and 16  $\mu$ g. per g. for the ox and rat respectively, figures which are of the same order of magnitude as those obtained by Mann *et al.* in experiments in which they claim that a considerable synthesis of acetylcholine has occurred. The conclusion thus seems inescapable that the acetylcholine formed in the presence of certain organic solvents has the same origin as that formed in aqueous media, i.e. that both are synthesized *in vitro*. We do not yet know the complete function of the organic solvent. One of its functions is, of course, to act as a vehicle for conveying the eserine into the tissue, but it probably also exerts some other action.

If it be agreed, as we think it must be, that the acetylcholine formed in the presence of chloroform or ether is synthesized *in vitro* and does not exist in a preformed condition in the tissue, it follows that any process capable of modifying the yield formed under these conditions can be regarded as legitimate evidence concerning the mechanism of the synthetic process. Now, with the exception of choline itself, which has not given entirely consistent results, we have so far discovered only two substances which modify the process to a considerable extent. These are sodium pyruvate and sodium acetoacetate. The former substance, when added to our chloroform preparation, produces a considerable inhibition of the formation of acetylcholine both at room temperature and at 37°. We conclude that sodium pyruvate cannot be a precursor of acetylcholine, and we might here also point out that its behaviour is scarcely consistent with the view that the acetylcholine formed in our chloroform preparation originates from a "preformed-precursor". Sodium acetoacetate, on the other hand, considerably increases the yield of acetylcholine both in the chloroform and ether preparation, the amount of additional acetylcholine for which it is responsible representing 4-5 times that present in the minced tissue before treatment with the solvent. The combined effect of incubation, after addition of ether-eserine, and addition of sodium acetoacetate is much greater than this and, as we have shown, is capable of raising the acetylcholine content to 23  $\mu$ g. per g., i.e. to about 15 times that originally present. We consider this to constitute substantial evidence that acetoacetic acid is, in fact, a precursor of acetylcholine. We admit that we should like to confirm this view by experiments with aqueous media, but we have so far failed to realize the necessary conditions. The second

precursor must, of course, be either choline itself or some derivative of choline. We do not propose, however, to discuss this point at present since, as pointed out above, our experiments with choline have not been entirely consistent.

#### SUMMARY

A study has been made of the formation of acetylcholine by brain tissue *in vitro* under various conditions. The amount formed when a suspension of minced ox brain in eserinizied Ringer is shaken for 2 hr. at 37° is approximately equal to that produced by grinding the minced tissue with chloroform-eserine and keeping it at room temperature for the same period.

Ether resembles chloroform in its ability to promote the formation of acetylcholine in brain tissue, but it is more efficient. In the case of ox brain, increases from 1.5  $\mu$ g. before to 17  $\mu$ g. per g. after treatment have been observed. The corresponding figures for rat brain tissue are 0.6  $\mu$ g. and 16  $\mu$ g. respectively.

The effect of ether is greater at 37° than at room temperature. The reverse holds for chloroform. An explanation of this difference is offered. Contrary to the statement of Mann *et al.* the amount of acetylcholine formed in the chloroform preparation at 0° is exceedingly small and is almost negligible compared with that produced at 37°.

Addition of sodium acetoacetate to the chloroform or ether preparations increases the yield of acetylcholine. Contrary to the statement of Mann *et al.* this increase also occurs in the chloroform preparation at room temperature.

When a suspension of minced rat brain in eserinizied Ringer is shaken in O<sub>2</sub> for 2 hr., acetylcholine to the extent of 18  $\mu$ g. per g. is formed. Addition of glucose to the suspension medium under conditions which have been carefully defined does not appreciably increase the yield. These results are contrary to those of Mann *et al.*

It is suggested (1) that acetoacetic acid is a precursor of acetylcholine, and (2) that the "preformed-precursor" of Mann *et al.* does not exist.

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# CIII. ACETYLCHOLINE METABOLISM IN THE CENTRAL NERVOUS SYSTEM

## THE EFFECTS OF POTASSIUM AND OTHER CATIONS ON ACETYLCHOLINE LIBERATION

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THE physiological evidence for the existence of a relationship between potassium ions and acetylcholine liberation in nervous structures [Brown & Feldberg, 1936; Feldberg & Guimaraes, 1936; Beznak, 1934] led us to examine the effects of changes of potassium ion concentration on the synthesis and liberation of acetylcholine in brain tissue *in vitro*. The results have thrown new light on the nature of the dynamic equilibria, involving acetylcholine metabolism, which exist in the central nervous system. The present communication is concerned with a description of these results and of their consequences.

### TECHNIQUE

*Estimation of acetylcholine.* The ester was estimated by measuring the contraction of the eserinated longitudinal muscle from the dorsum of a leech as recorded on a slowly revolving smoked drum. The contraction was compared with those caused by known quantities of acetylcholine chloride. All results are given in terms of acetylcholine chloride per g. tissue. Details of the estimation are given by Quastel *et al.* [1936].

*Preparation and examination of brain tissue.* The brain was carefully removed from the animal (rat, guinea-pig) as soon as possible after death. It was then either minced or sliced, the slices not being confined entirely to the cortex of the brain, but being taken from the entire cerebrum. Known quantities of the tissue (100–200 mg. wet weight)<sup>1</sup> were placed in suitable media in Warburg manometric vessels. The slices, 0.2–0.3 mm. thick, were bathed in Locke medium, allowed to drain thoroughly and weighed quickly on a torsion balance before being immersed in the medium in the Warburg flask. About 100 mg. wet weight of the tissue were placed in each flask, care being taken that there was an approximately equal distribution of the slices between the flasks. Possible inaccuracies due to the presence of different proportions of grey matter to white matter in the various flasks were eliminated by frequent repetition of the experiment, those results being accepted which had been consistently obtained after a number of experiments.

<sup>1</sup> It is important not to employ too large quantities of tissue under our experimental conditions, since not only may it be difficult to remove metabolites from such large quantities of tissue but the rates of O<sub>2</sub>-uptake may be so great as to be limited by the diffusion of oxygen into solution and not by the amount of tissue or by the concentration of metabolite. Under such conditions it is clearly impossible to carry out comparative experiments on the influence of metabolites, etc. on the rates of acetylcholine formation. We have found that amounts of the order given, e.g. 100 mg. wet weight, are the most convenient.

The vessels were filled with  $O_2$ , or a mixture of 95 %  $O_2$  and 5 %  $CO_2$ , or with other gases as will be indicated. The experimental period varied from 1 to 3 hr. at  $37^\circ$ , but was usually 1 hr., excluding an initial period of 15 min. allowed to enable the contents of the vessels to arrive at the temperature of the bath. The rates of  $O_2$ -uptake or of gas exchange by the tissue in each vessel were always noted, as these gave valuable indications of the metabolic activities of the tissues under the given experimental conditions. At the end of the experimental period, the acetylcholine activity of the contents of each manometric vessel was estimated.

*Media.* Solutions were made up to have an osmotic pressure approximately equal to 0.16 *M* NaCl. The final volume of the medium was made up to 3.0 ml. with 0.16 *M* NaCl after all other desired substances had been added. The pH was maintained at 7.4. The phosphate-Locke medium generally used consisted of NaCl, 0.13 *M*; KCl, 0.004 *M*;  $CaCl_2$ , 0.002 *M*; sodium phosphate buffer solution pH 7.4, 0.03 *M*. The bicarbonate-Locke medium, which was used in presence of an atmosphere of 95 %  $O_2$  + 5 %  $CO_2$ , consisted of NaCl, 0.13 *M*; KCl, 0.004 *M*;  $CaCl_2$ , 0.002 *M*;  $NaHCO_3$ , 0.025 *M*. To these media were added glucose (0.01 *M*), etc., as occasion demanded.

When minced brain tissue was used in a phosphate medium, the manometric vessels were filled with air.

Eserine sulphate (0.2 ml. 1/400) was added to the medium in the manometric vessel either at the commencement or at the termination of an experiment. It was frequently placed in the side tube of the vessel and tipped into the vessel at an appropriate time.

*Estimation of free acetylcholine.* The free acetylcholine is the amount found in the medium surrounding the tissue at the termination of an experiment. The slices were removed, after careful draining, from the contents of the vessel and placed in an eserinated-Locke medium. The contents of the vessel were then tested directly on the eserinated leech preparation, and the acetylcholine activity was estimated. When minced tissue was used, the contents of the manometric vessel were centrifuged and the acetylcholine activity of the clear centrifugate was estimated.

*Estimation of "combined" acetylcholine.* The slices from the manometric vessels, after being well washed in an eserinated Locke medium, were broken up with a glass rod and suspended in a solution of 2.1 ml. eserine-Locke medium, 0.5 ml. sodium phosphate (0.2 *M*) buffer solution pH 7.4 and sufficient *N* HCl to bring its pH to 2.0–3.0. After 30 min. at room temperature, the solution was made neutral (pH 7.4) by the addition of a few drops of *N* NaOH, the suspension was centrifuged and the acetylcholine activity of the clear centrifugate was estimated. The same treatment was accorded to minced tissue after this had been centrifuged and well washed with eserinated Locke medium.

This method of estimating "bound" or "combined" acetylcholine has been found to give quantitatively similar results to those obtained by trichloroacetic acid, chloroform or heat treatments.

*Estimation of "total" acetylcholine.* When it was unnecessary to make separate estimates of free and "combined" acetylcholine, the "total" (i.e. free + "combined") acetylcholine was determined by adding *N* HCl to the suspensions in the manometric vessels until the pH was 2.0–3.0, keeping them at room temperature for 30 min. and then making the solution neutral with *N* NaOH. The slices were well broken up with a glass rod before the solution was made acid. The suspensions were finally centrifuged and the "total" acetylcholine was estimated in the clear centrifugate.



*Preliminary remarks concerning acetylcholine synthesis in brain*

Quastel *et al.* [1936] showed that the formation of free acetylcholine by brain cortex slices is greatly increased when they are allowed to respire in a physiological medium containing eserine and either glucose, lactate or pyruvate. Since no such increased rate of formation of acetylcholine occurs when the slices are incubated in this medium under anaerobic conditions or when they are incubated in a medium free of a suitable metabolite (even if oxygen is freely available) it is evident that synthesis of acetylcholine takes place in brain tissue only under the proper physiological conditions. In the absence of eserine no acetylcholine can be detected, this being clearly due to the activity of the choline esterase present in the brain tissue.

It was then found by Mann, Tennenbaum & Quastel [1938] that there exists in brain tissue a combined form of acetylcholine which is pharmacologically inactive and which breaks down to yield free acetylcholine under a variety of conditions. It is stable under neutral conditions at 0°, but is less so at higher temperatures. Treatment with acid, e.g. at pH 3, releases free acetylcholine from the bound form at room temperature, but breakdown takes place at a higher pH, e.g. 6.0 or 6.5, at 37°. The substance releases free acetylcholine after treatment with a denaturing agent, e.g. after shaking with chloroform. It is apparently non-dialysable or diffusible and it has the properties of a protein complex. There can be little doubt that this substance is identical with the complex in brain tissue described by Corteggiani [1937] which on heating to 70° for 3 min. gives rise to acetylcholine. The action of alcohol, or of acid alcohol, in liberating free acetylcholine, described by Loewi [1937], is probably due to a denaturing action on "bound" acetylcholine. It has been thought for some time [see Gaddum, 1935] that acetylcholine found in tissue extracts may not be derived entirely from the freely diffusible ester in the tissue but that it may be present in the tissue as a comparatively stable precursor.

"Bound" acetylcholine was found by Mann, Tennenbaum & Quastel [1938] to be synthesized in brain tissue when the latter is allowed to respire in presence of glucose, lactate or pyruvate, but under anaerobic conditions, or in the absence of a suitable metabolite, no synthesis takes place. "Bound" acetylcholine is also synthesized by brain tissue under the proper physiological conditions in the absence of eserine, but, of course, in this case no free acetylcholine can be detected. The effect of glucose, lactate or pyruvate in securing synthesis of the "bound" acetylcholine is much greater than that due to added acetylcholine. It became clear that the experimental results were not consistent with the view that free acetylcholine is first formed and that this is adsorbed by the cell constituents to form "bound" acetylcholine. It seemed more probable that the synthesis of acetylcholine takes place through the intermediate formation of "bound" acetylcholine which we have therefore referred to as acetylcholine precursor. The main reasons for this conclusion are as follows.

(1) The formation of the complex is not greatly higher in the presence of eserine, which prevents the destruction of free acetylcholine, than in its absence [Mann, Tennenbaum & Quastel, 1938]. It would be expected that if the complex were formed secondarily by adsorption of free acetylcholine on cell structures the presence of eserine would greatly increase the formation of the complex because of the presence of an increased concentration of free acetylcholine.

(2) The rate of formation of the complex in the brain is more rapid than that of free acetylcholine. Typical results illustrating this statement are shown in Table I.

Table I

A fresh rat brain was minced finely and suspended in 5 vol. Locke medium in the absence of eserine. The suspension was divided into several equal parts which were then placed in Warburg vessels containing a phosphate-glucose (0.01 *M*)-eserine medium. Incubation was allowed to proceed in an atmosphere of air at 37°. The contents of the first vessel were analysed at once for free and combined acetylcholine; this gave the initial figures (i.e. preformed ester). The contents of the second vessel were analysed after 30 min. incubation, those of the third after 60 min. and so on.

Time min.	Free	Combined	Total acetylcholine μg./g.
0	0	<2.5	<2.5
30	2.5	6.3	8.8
60	3.7	7.5	11.2
120	7.5	7.5	15.0

Evidently the amount of "combined" acetylcholine rapidly reaches a limiting value whilst that of free acetylcholine slowly increases with time. This could occur if the complex were formed first, with subsequent breakdown to free acetylcholine, or if free acetylcholine were formed first with subsequent rapid adsorption to form the complex. Experiments designed to test the latter possibility have failed to show a sufficiently rapid rate of formation of the complex from added acetylcholine in presence of brain tissue [Mann, Tennenbaum & Quastel, 1938] and the conclusion must therefore be drawn that the complex, and not the free, acetylcholine is first produced.

Trethowie [1938] argues that the complex is an association of acetylcholine with cell debris. Doubtless this is true, but since it is agreed that all but traces of acetylcholine cannot be diffusible in brain tissue and are therefore "bound", and since experiment [Mann, Tennenbaum & Quastel, 1938] has shown that the ester is not adsorbed by a variety of brain tissue components, it follows that the complex must be an association of acetylcholine with a specific constituent of the debris. It is reasonable to regard the complex as a definite chemical entity until evidence to the contrary is forthcoming.

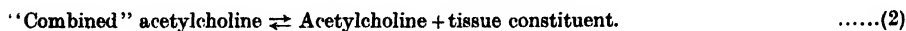
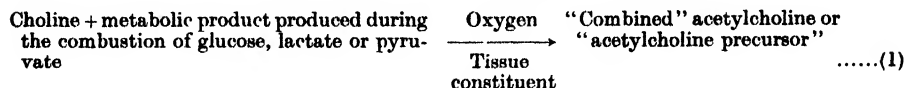
That some formation of the complex takes place when acetylcholine is added to brain tissue initially devoid of it is apparent from the experiments of Mann, Tennenbaum & Quastel [1938] and of Corteggiani [1938]. It therefore seems that an equilibrium exists in the cell between the complex and the free acetylcholine. This is borne out by the fact that in presence of eserine there is a less rapid rate of disappearance of the complex than in its absence (see also Corteggiani [1938]). Typical results indicating this fact are shown in Table II. Eserine, by allowing accumulation of free acetylcholine to occur, enables also some formation of the bound form to take place and thus has the effect of stabilizing the complex.

Table II

A rat brain was minced and suspended in 5 vol. saline. This was divided into several equal parts and one part was examined immediately for "bound" acetylcholine. Each of the remaining parts was placed in an appropriate medium in an atmosphere of nitrogen and incubation was allowed to proceed for 2½ hr. at 37°. After this period the contents of the vessels were analysed for the bound ester.

Medium	"Combined" acetylcholine μg./g.
None	(initial value) 2.8
Phosphate-Locke	1.0
Phosphate-Locke-eserine	2.6

The following reaction schemes account for the results which have been obtained so far (see Mann, Tennenbaum & Quastel [1938]):



Reaction (1) is normally rapid compared with reaction (2). The quantity of free acetylcholine in the cell must normally be low owing to the activity of the choline esterase present, the amount present being determined by the conditions of the dynamic equilibrium set up.

The conclusion that glucose, or a breakdown product of glucose, is important for the synthesis of acetylcholine has received confirmation from MacIntosh's [1938] perfusion experiments carried out on the cat's superior cervical ganglion.

#### *Effects of $K^+$ on the synthesis of acetylcholine in brain*

Experiments with rat brain slices show that the addition of  $K^+$  to the medium in which the tissue is respiring may bring about a very large increase in the rate of acetylcholine formation. Typical results are shown in Table III. The effect of the addition of  $0.027 M K^+$  may result in the formation of acetylcholine to the extent of over  $40 \gamma/g.$ <sup>1</sup>

Table III

Rat brain slices in glucose ( $0.01 M$ )-bicarbonate-Locke-eserine medium. KCl was added to the medium, with a corresponding decrease in NaCl to preserve isotonicity. 95%  $O_2$  + 5%  $CO_2$ . 1 hr.  $37^\circ$ .

Exp. ...	Total acetylcholine formed $\mu g./g.$				
	A	B	C	D	E
Medium with no extra KCl	12.7	10.4	12.0	20.0	21.3
+ $0.0135 M$ KCl	16.5	—	—	—	—
+ $0.027 M$ KCl	37.5	41.0	34.0	41.0	45.0
+ $0.054 M$ KCl	—	—	15.0	—	—
+ $0.11 M$ KCl	—	4.0	—	—	—
+ $0.13 M$ KCl	4.3	—	—	—	—

The presence of a much greater  $[K^+]$  brings about, however, a fall in the rate of formation of acetylcholine. Concentrations of the order of  $0.1 M K^+$  inhibit the synthesis of the ester.

It is evident that the accelerating action of  $0.027 M$  KCl on the rate of formation of acetylcholine is due to  $K^+$  itself and not to a fall in the  $[Na^+]$ . A fall in the NaCl concentration to the extent of  $0.027 M$  has little or no effect on the rate of formation of acetylcholine.

Analysis of the "total" acetylcholine formed in presence of excess  $K^+$  shows that the increase of acetylcholine formation lies entirely with the free acetylcholine, the amount of "combined" ester being usually decreased. These results are shown in Table IV.

If eserine is added to the medium at the termination of an experiment, it is found that the action of added  $K^+$  is to decrease the formation of acetylcholine.

<sup>1</sup> Control experiments have been carried out to show that the amount of  $K^+$  introduced with the medium into the solution bathing the leech does not itself cause a contraction or affect that due to acetylcholine.

Table IV

Rat brain slices. Glucose-bicarbonate-Locke-eserine medium. 95% O<sub>2</sub> + 5% CO<sub>2</sub>. 1 hr. 37°.

	Acetylcholine formed $\mu\text{g./g.}$			
	Exp. A		Exp. B	
	Free	Combined	Free	Combined
Medium + no added KCl	3.7	9.0	3.2	12.8
+ 0.027 M KCl	30.0	7.5	20.1	6.6

This is due to the fact that when eserine is added at the end of an experiment, the acetylcholine estimated is almost wholly "bound" acetylcholine. These facts are shown in Table V.

Table V

Rat brain slices. Glucose-bicarbonate-Locke medium. 95% O<sub>2</sub> + 5% CO<sub>2</sub>. 1 hr. 37°.

	"Total" acetylcholine formed $\mu\text{g./g.}$	
	Eserine added at beginning of the exp.	Eserine added at the end of the exp.
Medium + no added KCl	20.0	17.0
+ 0.027 M KCl	41.0	11.0

The action of K<sup>+</sup> in increasing the formation of free acetylcholine in brain slices takes place also in phosphate media, but the effect is less marked than in bicarbonate media (Table VI).

Table VI

Rat brain slices. Phosphate-glucose-Locke-eserine medium. O<sub>2</sub>. 1 hr. 37°.

	Acetylcholine formed $\mu\text{g./g.}$	
	Free	Combined
Medium + no extra KCl	3.5	8.6
+ 0.027 M KCl	10.0	7.0

*The stimulating action of K<sup>+</sup> is confined to a "synthesizing" medium*

The stimulating effect of K<sup>+</sup> on acetylcholine formation is not confined to glucose. It takes place also in a pyruvate medium in which synthesis of acetyl-

Table VII

Rat brain slices. O<sub>2</sub> or 95% O<sub>2</sub> + 5% CO<sub>2</sub>. 1 hr. 37°.

Exp. A	Medium	Acetylcholine formed $\mu\text{g./g.}$	
		Free	Combined
	Phosphate-Locke-eserine-pyruvate (0.01 M)	3.5	8.9
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.027 M KCl	9.6	6.8
	Bicarbonate-Locke-eserine-pyruvate (0.03 M)	6.8	13.7
	Bicarbonate-Locke-eserine-pyruvate (0.03 M) + 0.027 M KCl	30.7	9.2
		Total acetylcholine formed $\mu\text{g./g.}$	
Exp. B			
	Phosphate-Locke-eserine-pyruvate (0.01 M)		11.2
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.011 M KCl		12.3
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.021 M KCl		14.7
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.04 M KCl		15.7
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.069 M KCl		12.2
Exp. C			
	Bicarbonate-Locke-eserine		3.1
	Bicarbonate-Locke-eserine + 0.027 M KCl		2.5

choline occurs. It does not take place, however, in the absence of a substrate, where no synthesis obtains. As in the case of glucose, the effect of  $K^+$  on acetylcholine synthesis in a pyruvate medium is less in a phosphate medium than in bicarbonate, and the accelerating action lies entirely with free acetylcholine. These results are shown in Table VII.

*The antagonistic actions of  $Ca^{++}$  and  $Mg^{++}$*

The addition of  $Ca^{++}$  to a glucose-bicarbonate-Locke medium decreases the rate of formation of acetylcholine by brain slices and neutralizes the accelerating action of added  $K^+$ .  $Mg^{++}$  has a similar neutralizing action. Typical results are shown in Table VIII.

Table VIII

Rat brain slices. Bicarbonate-glucose-Locke-eserine media. 95%  $O_2$  + 5%  $CO_2$ . 1 hr. 37°.

	Exp. A	Total acetylcholine formed $\mu g./g.$
Medium		21.3
+ 0.027 M KCl		45.0
+ 0.02 M $CaCl_2$		10.4
+ 0.027 M KCl + 0.02 M $CaCl_2$		15.0
+ 0.027 M KCl + 0.015 M $CaCl_2$		15.0
	Exp. B	
Medium		18.2
+ 0.027 M KCl		40.5
+ 0.02 M $MgCl_2$		20.2
+ 0.027 M KCl + 0.02 M $MgCl_2$		18.8

*Action of  $Rb^+$  and  $Cs^+$*

The effects of  $K^+$  and  $Ca^{++}$  on acetylcholine liberation in brain slices are in complete accord with the results of Brown & Feldberg [1936] on the action of these ions on the liberation of acetylcholine from the perfused superior cervical ganglion of the cat. These authors further showed that  $Rb^+$ , and to a lesser degree  $Cs^+$ , have effects similar to  $K^+$ .

$Rb^+$  and  $Cs^+$  have effects similar to  $K^+$  on the liberation of acetylcholine by brain slices (see Table IX),  $Cs^+$  being rather less active than  $K^+$  or  $Rb^+$ .

Table IX

Rat brain slices. Bicarbonate-glucose-Locke-eserine medium. 95%  $O_2$  + 5%  $CO_2$ . 1 hr. 37°.

	Acetylcholine formed $\mu g./g.$	
	Free	Combined
Medium	5.2	13.6
+ 0.027 M KCl	27.8	5.0
+ 0.027 M RbCl	27.9	5.0
+ 0.027 M CsCl	23.0	4.7

*Interpretation of the  $K^+$  effect*

The action of  $K^+$  might be explained as being due to a catalysing action of these ions on the rate of breakdown of the combined acetylcholine into the free ester. It is difficult to understand, however, with this explanation how  $Ca^{++}$  brings about its antagonistic effects. It is equally difficult to understand why the addition of 0.027 M KCl to a medium should directly influence the breakdown of the complex into free acetylcholine when it is remembered that  $[K^+]$  in the nerve cell may reach a concentration of 0.1 M.

It is reasonable to assume that  $\text{Ca}^{++}$  acts by neutralizing some effect of added  $\text{K}^+$  on cell permeability, such an assumption being in accordance with the known physiological behaviour of these ions.

On this hypothesis the addition of  $\text{K}^+$  brings about a change in nerve cell permeability so that free acetylcholine diffuses into the outer medium at a relatively rapid rate.  $\text{Ca}^{++}$  has an antagonistic effect.

*Acetylcholine formation in Locke and saline media*

It would be expected from the observation that the presence of  $\text{Ca}^{++}$  retards the liberation of acetylcholine, that an increased rate of formation of free acetylcholine will take place when respiration of brain slices occurs in a  $\text{Ca}$ -free medium.

This doubtless accounts, at any rate partly, for the fact that when brain slices respire in a Locke medium a smaller quantity of free acetylcholine is produced than when respiration occurs in a medium free from  $\text{K}^+$  and  $\text{Ca}^{++}$ . The  $[\text{K}^+]$  in the Locke medium is probably insufficient to neutralize the retarding action of the  $\text{Ca}^{++}$  present. Moreover, the effects of adding 0.027 *M*  $\text{KCl}$  to a saline medium (i.e. free from  $\text{Ca}^{++}$ ) on the liberation of free acetylcholine by brain slices is far less marked than when this concentration of  $\text{K}^+$  is added to a Locke medium.<sup>1</sup> These results are shown in Table X, where it will be observed that the differences found between the rates of free acetylcholine formation in Locke and saline media occur in both phosphate and bicarbonate media. The phenomenon occurs also in pyruvate media. It should be noted that the amount of "combined" acetylcholine is decreased under those conditions where an increase in the free acetylcholine takes place.

Table X

Rat brain slices.  $\text{O}_2$  or 95%  $\text{O}_2$  + 5%  $\text{CO}_2$ . 1 hr. 37°.

Exp. A	Medium	Acetylcholine formed $\mu\text{g./g.}$	
		Free	Combined
	Glucose- eserine-Locke-bicarbonate	3	17
	Glucose- eserine-NaCl-bicarbonate	14.7	11
Exp. B			
	Glucose- eserine-Locke-phosphate	3.5	8.6
	Glucose- eserine-Locke-phosphate 0.027 <i>M</i> $\text{KCl}$	9.6	6.8
Exp. C			
	Glucose- eserine-NaCl-phosphate	6.5	8.0
	Glucose- eserine-NaCl-phosphate 0.027 <i>M</i> $\text{KCl}$	6.5	8.0
Exp. D			
	Pyruvate (0.01 <i>M</i> )- eserine-Locke-phosphate	3.5	8.9
	Pyruvate- eserine-NaCl-phosphate	6.5	8.0

*Effect of  $\text{K}^+$  on acetylcholine formation by minced brain tissue*

It is an interesting fact that the marked increase in the rate of formation of "total" acetylcholine effected by  $\text{K}^+$  on intact brain slices does not take place with minced brain tissue. The presence of  $\text{K}$ , however, brings about a fall in the "combined" acetylcholine with a corresponding increase in the amount of free acetylcholine. These results may be seen in Table XI.

<sup>1</sup> In fact this concentration of  $\text{K}^+$  has been observed sometimes to inhibit the synthesis of total acetylcholine if  $\text{Ca}$  is absent from the medium.

Table XI

Exp. A. A rat brain was minced finely and suspended in 3 vol. saline and divided into several equal parts, each of which was added to the appropriate medium in a Warburg flask. Medium: glucose-bicarbonate-eserine-Locke. When KCl was present, the NaCl was decreased correspondingly to preserve isotonicity. 95% O<sub>2</sub> + 5% CO<sub>2</sub>. 1 hr. 37°.

	Acetylcholine formed $\mu\text{g./g.}$		
	Free	Combined	Total
Medium + no added KCl	7.5	6.0	13.5
+ 0.027 M KCl	11.2	3.0	14.2
+ 0.053 M KCl	5.6	1.9	7.5
+ 0.08 M KCl	3.0	1.5	4.5

Exp. B. As in exp. A but with the following medium: phosphate-eserine-Locke. Air. 1 hr. 37°.

	Acetylcholine formed $\mu\text{g./g.}$		
	Free	Combined	Total
Medium	3.8	7.0	10.8
+ 0.07 M KCl	6.4	3.0	9.4
+ glucose	7.9	11.2	19.1
+ glucose + 0.07 M KCl	7.6	5.3	12.9

#### *Action of K<sup>+</sup> on "combined" acetylcholine*

To perceive more clearly the effects of K<sup>+</sup> experiments were carried out with brain slices and minced brain under conditions where synthesis of the ester was reduced to a minimum.

In these experiments minced brain tissue was allowed to respire in a phosphate-glucose-eserine medium in an atmosphere of air for 1½ hr. at 37° to build up a relatively high concentration of "combined" acetylcholine. The tissue was then centrifuged, the clear centrifugate containing the free acetylcholine was discarded and the tissue deposit containing the "combined" ester was immersed in a glucose-free Locke-eserine medium. The effect of added K<sup>+</sup> on the rate of breakdown of the "combined" ester under such conditions, where little or no synthesis of further acetylcholine took place, was investigated. The results showed that the effect of added K<sup>+</sup> was always to reduce the amount of "combined" ester and to increase the free acetylcholine. When the experiments with added K<sup>+</sup> were carried out under anaerobic conditions, the increase in the free acetylcholine was equal to the decrease in the "combined" acetylcholine, the "total" acetylcholine being unaffected by K<sup>+</sup>. Under aerobic conditions it was usually found that the "total" acetylcholine was decreased by the addition of K<sup>+</sup>. This was doubtless due to the fact that some synthesis of acetylcholine usually takes place under aerobic conditions when minced tissue is used owing to the presence of small quantities of metabolites which cannot be washed out, and the addition of K<sup>+</sup> inhibits this synthesis. The effect, however, of K<sup>+</sup> in causing "combined" acetylcholine to break down to form free acetylcholine is as evident under aerobic conditions as it is under anaerobic conditions. Typical results are shown in Table XII. In this table, too, is shown the effect of added K<sup>+</sup> on the "combined" (preformed) acetylcholine of brain slices incubated in an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub> in a glucose-free medium at 25° when very little synthesis takes place. The usual action of K<sup>+</sup> in causing breakdown of the "combined" ester will be observed.

Table XII

Exp. A. Preliminary synthesis of "combined" acetylcholine by minced brain in phosphate-glucose-Locke-eserine medium for 1½ hr. at 37°. Tissue residue, after centrifuging, was divided into several parts and placed in Warburg vessels containing phosphate- or bicarbonate-NaCl-eserine media with different concentrations of KCl. Conditions either aerobic (air or 95% O<sub>2</sub> + 5% CO<sub>2</sub>) or anaerobic (95% N<sub>2</sub> + 5% CO<sub>2</sub>). 1 hr. 37°.

Medium. (Eserine always present)	Acetylcholine found $\mu\text{g./g.}$		
	Free	Combined	Total
Exp. 1			
N <sub>2</sub> -bicarbonate-NaCl	4.3	6.0	10.3
N <sub>2</sub> -bicarbonate-NaCl + 0.08 M KCl	6.4	3.7	10.1
N <sub>2</sub> -bicarbonate-NaCl + 0.12 M KCl	6.4	3.7	10.1
Exp. 2			
O <sub>2</sub> -bicarbonate-NaCl	3.8	10.5	14.3
O <sub>2</sub> -bicarbonate-NaCl + 0.04 M KCl	5.5	4.5	10.0
O <sub>2</sub> -bicarbonate-NaCl + 0.08 M KCl	6.4	3.3	9.7
O <sub>2</sub> -bicarbonate-NaCl + 0.12 M KCl	6.4	3.3	9.7
Exp. 3			
Air-phosphate-NaCl	3.0	8.1	11.1
Air-phosphate-NaCl + 0.12 M KCl	6.5	4.5	11.0
Exp. 4			
Air-phosphate-NaCl	3.0	13.5	16.5
Air-phosphate-NaCl + 0.08 M KCl	5.9	7.5	13.6
Air-phosphate-NaCl + 0.12 M KCl	6.9	6.7	13.6
Exp. 5			
N <sub>2</sub> -phosphate-NaCl	4.3	5.3	9.6
N <sub>2</sub> -phosphate-NaCl + 0.12 M KCl	6.0	3.7	9.7
Exp. B. Rat brain slices. Bicarbonate-eserine-Locke. 95% O <sub>2</sub> + 5% CO <sub>2</sub> . 1 hr. 25°.			
Medium	0.9	2.6	—
Medium + 0.027 M KCl	1.5	1.5	—

*Permeability of the nerve cell to acetylcholine in presence of K<sup>+</sup>*

The effect of K<sup>+</sup> in causing breakdown of the "combined" acetylcholine may be due to a direct action on the complex, as stated earlier; but since such an explanation fails to account for the antagonistic action of Ca<sup>++</sup> on the liberation of free acetylcholine, an effect on cell permeability is considered to afford a more reasonable explanation of the phenomenon.

If K<sup>+</sup> effects a liberation of free acetylcholine by causing a change in permeability of the nerve cell so that free acetylcholine diffuses out more rapidly than in the absence of K<sup>+</sup>, it follows that there must exist normally within the cell a certain amount of diffusible or free acetylcholine. In brain tissue, in the presence of eserine, the "combined" acetylcholine remains comparatively stable. Yet it is able to break down normally to form free acetylcholine—otherwise changes of permeability alone could effect no increased liberation of the ester. The most obvious manner to reconcile these facts is to assume that an equilibrium exists in the cell between the free and "combined" forms of the ester. A disturbance of this equilibrium due to diffusion of acetylcholine through the cell membrane, when the permeability is increased by added K<sup>+</sup>, will result in a decrease in the cell of the amount of "combined" acetylcholine. This, presumably, is what takes place in brain tissue when K<sup>+</sup> is added and when no synthesis is occurring. Under optimal respiratory conditions, however, the "combined" acetylcholine is being rapidly synthesized, its amount being determined by the amount of tissue constituent with which the ester is combined. The rate of its breakdown to free acetylcholine is determined by equilibrium





In these experiments a known quantity, e.g. 50  $\mu\text{g.}$ , of acetylcholine was added to brain slices respiring in a bicarbonate-glucose-Locke-eserine medium, with and without the addition of 0.027  $M$  KCl. It was found that in presence of this quantity of acetylcholine the presence of the added  $\text{K}^+$  effected little or no increase in the "total" acetylcholine. It was also easy to show that the presence of added acetylcholine greatly decreased the synthesis of acetylcholine, this being most clearly observable in the presence of 0.027  $M$  KCl. A concentration of acetylcholine of 10  $\mu\text{g./ml.}$  retarded the synthesis of acetylcholine by 40 % in the presence of 0.027  $M$  KCl and had no effect on the synthesis in the absence of  $\text{K}^+$ . Typical results are shown in Table XIV.

Table XIV

Rat brain slices. Bicarbonate-glucose-eserine-Locke medium. 95 %  $\text{O}_2$  + 5 %  $\text{CO}_2$ . 1 hr. 37°.

Exp. A	"Total" acetylcholine found	"Total" acetylcholine synthesized
Medium	20	20
+ 50 $\mu\text{g.}$ acetylcholine	66	16
+ 0.027 $M$ KCl	33	33
+ 0.027 $M$ KCl + 50 $\mu\text{g.}$ acetylcholine	66	16
Exp. B		
Medium	20	20
+ 30 $\mu\text{g.}$ acetylcholine	50	20
+ 0.027 $M$ KCl	40.5	40.5
+ 0.027 $M$ KCl + 30 $\mu\text{g.}$ acetylcholine	54	24
Exp. C		
Medium	23	23
+ 50 $\mu\text{g.}$ acetylcholine	64	14
+ 0.027 $M$ KCl	50	50
+ 0.027 $M$ KCl + 50 $\mu\text{g.}$ acetylcholine	66	16

The inhibitory action of free acetylcholine on the synthesis of acetylcholine points to the existence of an equilibrium as expressed in the reaction scheme<sup>1</sup> (3). Moreover, the facts that this inhibition is observable in presence of excess of  $\text{K}^+$  and that it occurs to a much less extent in the absence of  $\text{K}^+$  are confirmatory of the conclusion that potassium greatly increases the permeability of the brain cell to acetylcholine.

#### *Effects of $\text{NH}_4^+$*

The addition of  $\text{NH}_4^+$  to a medium in which brain slices are respiring has apparently little effect on the rate of synthesis of acetylcholine until the  $[\text{NH}_4^+]$  reaches a value of approximately 0.05  $M$ , when a decided inhibition takes place. Analysis, however, of the acetylcholine formed in the presence of  $\text{NH}_4^+$  shows a considerable drop in the amount of "combined" acetylcholine and a corresponding increase in that of the free acetylcholine. It is evident that an effect similar to that of  $\text{K}^+$  takes place with  $\text{NH}_4^+$ , but that in contrast to  $\text{K}^+$ ,  $\text{NH}_4^+$  ions at relatively low concentrations have highly inhibitory effects on the synthesis of acetylcholine by brain slices. This inhibitory action on acetylcholine synthesis is clearly shown when  $\text{NH}_4^+$  is added together with  $\text{K}^+$  to brain slices; the increased rate of synthesis found with  $\text{K}^+$  is practically eliminated. Typical results are shown in Table XV.

It is most likely that  $\text{NH}_4^+$ , like  $\text{K}^+$ , causes an increased permeability of the brain cells for acetylcholine and would, like  $\text{K}^+$ , have accelerating effects

<sup>1</sup> Conceivably acetylcholine may inhibit by competing with choline for a receptor group in the synthesizing enzyme.

Table XV

Rat brain slices. Bicarbonate-glucose-eserine-Locke medium. 95% O<sub>2</sub> + 5% CO<sub>2</sub>.

		Acetylcholine formed, $\mu\text{g.}$		
		Free	Combined	Total
Exp. A				
	Medium	4.9	10.1	15.0
	+ 0.027 <i>M</i> KCl	31.8	4.2	36.0
	+ 0.027 <i>M</i> NH <sub>4</sub> Cl	10.6	3.1	13.7
	+ 0.027 <i>M</i> KCl + 0.027 <i>M</i> NH <sub>4</sub> Cl	7.6	1.9	9.5
Exp. B				
	Medium	—	—	17.5
	+ 0.013 <i>M</i> NH <sub>4</sub> Cl	—	—	20.0
	+ 0.027 <i>M</i> NH <sub>4</sub> Cl	—	—	17.0
	+ 0.053 <i>M</i> NH <sub>4</sub> Cl	—	—	7.0

on the formation of total acetylcholine were it not for its secondary inhibitory action on acetylcholine synthesis. It is very suggestive that the glutamic acid-glutamine system, which would act as an effective detoxicating process so far as NH<sub>4</sub><sup>+</sup> in the brain is concerned, exists in relatively large amount in the central nervous system [Krebs, 1935]. The toxicity of NH<sub>4</sub><sup>+</sup> may be due to its competition with choline for the enzyme synthesizing acetylcholine. It has already been shown by Mann, Woodward & Quastel [1938] that NH<sub>4</sub><sup>+</sup> competes with choline for the choline oxidase system in liver.

It is worthy of note that NH<sub>4</sub><sup>+</sup>, like K<sup>+</sup>, greatly increases the aerobic glycolysis of brain tissue [Weil-Malherbe, 1938], an effect which has been ascribed to possible altered permeabilities of the cell membrane [Dickens & Greville, 1935].

*A suggestion as to the chemical nature of "combined" acetylcholine*

There is little evidence as yet which throws any light on the composition of the acetylcholine precursor or of "bound" acetylcholine, beyond the facts stated in our earlier paper [1938] which indicate that the substance is a protein complex. We would like, however, to make the suggestion that the complex is a compound of acetylcholine with its synthesizing enzyme. It is most probable that, when acetylcholine is first formed, its synthesis occurs at the surface of an enzyme, and it is not unreasonable to consider that a definite quantity of the compound between acetylcholine and the enzyme which synthesizes it exists normally in the cell. Such a view would involve the conclusion that the complex will only be found in tissues which are capable of synthesizing acetylcholine and so far as we know at present this is the case. The amount of "bound" acetylcholine present in the cell is also not irreconcilable with this view. The amount of complex present in brain under our experimental conditions, when synthesis is actively taking place and with eserine present, is of the order of 14  $\mu\text{g./g.}$ , this being reckoned as acetylcholine chloride. Assuming that the complex is a union of one molecule of protein (say of mol. wt. 34,000) and of one molecule of acetylcholine, the amount of complex present in brain corresponding to 14  $\mu\text{g./g.}$  would be approximately 2.6 mg. per g. wet weight of tissue. Such a value would not be unexpected.

#### SUMMARY

1. The addition of potassium ions (0.027 *M*) to a medium containing eserine and in which intact brain slices are respiring brings about a large increase in the rate of formation of acetylcholine. In a bicarbonate-glucose medium, or a

bicarbonate-pyruvate medium the total acetylcholine formed may reach a value of 40  $\mu$ g. per g. wet weight of tissue. High concentrations of potassium ions inhibit the synthesis of acetylcholine by brain tissue.

2. The accelerating effect of potassium ions does not take place in a substrate-free medium.

3. The increase in acetylcholine formation effected by potassium lies entirely with the free acetylcholine. The amount of "combined" acetylcholine is either unchanged or diminished.

4. The accelerating action of potassium ions is neutralized by the addition of calcium or magnesium ions.

5. Rubidium, and to a smaller extent caesium, ions have effects similar to those of potassium.

6. The addition of potassium ions to minced brain tissue does not have such a marked effect on acetylcholine synthesis as in the case of intact brain slices.

7. The addition of potassium ions to either minced brain tissue or intact brain slices, under conditions when synthesis of acetylcholine is not taking place, is to bring about a fall in the amount of "combined" acetylcholine with a corresponding (equal) increase in the amount of free acetylcholine. This occurs under anaerobic, as well as under aerobic, conditions.

8. The effect of potassium ions is less marked in a phosphate medium than in a bicarbonate medium.

9. The effect of potassium ions is to increase the permeability of the nerve cell, so that acetylcholine diffuses through the cell membrane at a greater rate than in the absence of potassium.

10. An equilibrium exists in the cell between free acetylcholine and "combined" acetylcholine. The addition of acetylcholine to a medium in which brain slices are respiring depresses the synthesis of acetylcholine by the tissue. This can only clearly be seen after the addition of potassium ions.

11. Ammonium ions act similarly to potassium ions in bringing about a breakdown of "combined" acetylcholine with a corresponding increase of free acetylcholine. They have a highly inhibitory action on acetylcholine synthesis by brain slices.

12. Reaction schemes accounting for these facts are described.

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# CIV. THE FERMENTATION PROCESS IN TEA MANUFACTURE

## II. SOME PROPERTIES OF TEA PEROXIDASE

### III. THE MECHANISM OF FERMENTATION

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## II. SOME PROPERTIES OF TEA PEROXIDASE

IN the first paper on this subject [Roberts & Sarma, 1938] it was shown that during fermentation tea tannin was oxidized by peroxidase and  $H_2O_2$ . Since this paper was written a considerable amount of experimental data on tea peroxidase has been accumulated from which it is possible to draw some conclusions as to the mechanism of the fermentation process.

### *Peroxidase content of green leaf*

Peroxidase activities are determined by the method previously described. Except where otherwise stated, these activities are expressed in terms of so many indophenol units (I.U.) per g. dry weight of tissue and are determined using 10 g. samples of leaf. There are great variations in the activities of single shoots from the same bush, and in representative samples from different bushes in the same plot. Thus eight single shoots from one bush, weighing from 0.24 to 0.63 g., had peroxidase activities of 511, 549, 607, 710, 808, 880, 941 and 1040 I.U. Similarly the average peroxidase contents of each of 12 bushes selected at random from a small plot of tea were 218, 237, 250, 274, 278, 298, 311, 311, 314, 322, 385, 409 I.U. Despite these variations, if the leaf from a whole plot amounting to about 20–30 lb. is bulked, and 10 g. samples are taken for analysis, quite good replication is obtained. The mean activity of four such samples gives quite an accurate measure of the average peroxidase activity of leaf from the whole of a plot. It was rare in a whole season's observations that any one sample varied by more than 10% from the mean of the four and the average standard error of the mean was about 3%.

There are marked variations from week to week in the peroxidase activity of leaf plucked from the same plot over the whole manufacturing season as is shown in the curve (Fig. 1). The highest recorded value is 950 I.U. and the lowest 300 I.U. Despite these variations there is little or no change in the rate of fermentation, from which it follows that peroxidase activity is not the sole factor governing this rate. This seasonal curve for peroxidase activity falls into three well-defined parts, a period lasting till 20 June 1938 when peroxidase activity falls rapidly to a minimum, an intermediate period lasting until 3 October 1938 when the activity remains at a fairly constant level, and a final period where activity rises to a maximum of 757 I.U. and then falls steadily to

values of about 500 I.U. It is interesting to note that these three periods correspond closely with three quite sharply contrasted types of teas. The first period corresponds with the early "second flush" teas characterized by good quality, the second with the typical "rains" teas of no marked flavour and less strong liquoring properties, and the third with "autumnals". It is possible that the variations in peroxidase content are an index of physiological conditions of the leaf associated with these three types of teas.

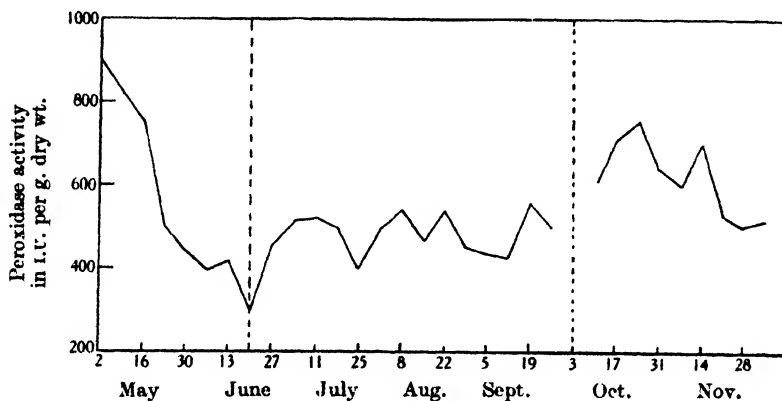


Fig. 1. Seasonal variation of peroxidase activity.

#### *Influence of variety on peroxidase content*

Certain "jats"<sup>1</sup> of tea are characterized by a relatively slow rate of fermentation. Thus Kharikatia and Singlo leaf ferment more slowly than Betjan. The tannin content of all three varieties is much the same, so that it would seem quite likely that an enzyme deficiency would account for any slowness in fermenting. However, differences in peroxidase content are not big enough to account for these differences, as the following figures show.

Table I

	Betjan	Kharikatia
	I.U.	I.U.
27. vi. 38	465	480
4. vii. 38	521	419
22. viii. 38	552	523

It follows that peroxidase and tannin contents alone do not determine the rate at which fermentation proceeds. Further evidence for this is a complete lack of correlation between the strength of liquors in teas manufactured from single bushes and the peroxidase content of the green leaf from these bushes. If peroxidase content did determine the rate of fermentation some such correlation might be expected. The author is indebted to Dr Wight for much of the data for these comparisons.

#### *Peroxidase content of different parts of the shoot*

Shoots consisting of three leaves and a bud were plucked and the peroxidase contents of the various parts determined.

<sup>1</sup> A "jat" of tea is best defined as the progeny of the seed obtained from a commercial seed-garden. There is such complete hybridization nowadays that a pure line variety cannot be obtained.

Table II

	Peroxidase content I.U. per g. dry wt.
Bud	268
1st leaf	486
2nd leaf	556
3rd leaf	574
Stalk	493

The increase of peroxidase content with the size of the leaf confirms earlier findings that old leaf, too coarse for plucking, has a higher peroxidase activity than new shoots. Despite its lower peroxidase content, bud ferments at the same rate or even faster than the first leaf when these parts of the shoot are taken separately. This has been shown both by the rate of decrease in the tannin titre and, manometrically, by measuring the rate of oxygen uptake.

#### *Properties of tea peroxidase*

##### *Temperature coefficient.*

A series of peroxidase activity determinations was made, using the same solution of peroxidase, over a range of temperatures from 15° to 37°. A straight line relationship was found and the activity  $P_T$  at any temperature  $T$  was related to  $P_0$  the activity at 25° by the equation

$$P_T = P_0 [1 + 0.02 (T - 25)].$$

The low value for the temperature coefficient is perhaps due to increasing competition at higher temperatures of the catalase, always present, for the  $H_2O_2$ .

##### *Inhibitors.*

The effect of different concentrations of various inhibitors is recorded below:

Table III

NaF	I.U.	$C_6H_5NH.NH_2$	I.U.	Ascorbic acid	I.U.	$N_2H_4$	I.U.
Nil	581	Nil	581	Nil	743	Nil	542
<i>M</i> /200	196	<i>M</i> /2000	96	<i>M</i> /20	Nil	<i>M</i> /2500	542
<i>M</i> /50	143	<i>M</i> /500	Nil	—	—	<i>M</i> /1000	439
<i>M</i> /20	104	<i>M</i> /100	Nil	—	—	<i>M</i> /500	379
—	—	—	—	—	—	<i>M</i> /50	130

Despite the complete inhibition of peroxidase activity by low concentrations of phenylhydrazine, this inhibitor has very little effect on the rate of fermentation. There is a slight initial inhibition, after which fermentation proceeds at its normal rate. The phenylhydrazine is probably fixed by the carbohydrates present, after which it can exert no further inhibitory effect. Phenylhydrazine inhibition of peroxidase is reversible, judging by the initial inhibition and the complete recovery of the fermenting system from the effects of the inhibitor.

##### *Freed from oxidase.*

A peroxidase preparation is always liable to show a slightly positive oxidase reaction if traces of  $H_2O_2$  are present. A more specific test for oxidase is its ability to catalyse oxidation of catechol by atmospheric oxygen. Manometric experiments fail to show any  $O_2$  uptake when catechol is acted upon by a tea peroxidase preparation. It may therefore be concluded that the tea leaf is free from polyphenol oxidase.

*Increase of peroxidase during withering*

The increase in peroxidase content of green leaf after withering, mentioned in the preceding communication, has been repeatedly confirmed. Loss of moisture alone is not responsible for this increase, as leaf kept in a closed vessel, in which the air is maintained at a 100 % relative humidity, still increases in peroxidase activity although not to the same extent as it does in a normal wither. The withering process itself therefore has some effect in determining the increase in peroxidase.

Table IV

Fresh leaf 76.82 % moisture	Leaf kept 24 hr. at 100 % R.H.	Withered leaf 68.10 % moisture
I.U.	I.U.	I.U.
420	603	614
478	588	635
449	603	699
449	588	667
Mean 449	596	654

Four 10 g. samples for peroxidase activity determination were taken in each case.

Many experiments have been carried out in which the increase of peroxidase has been determined under varying conditions of wither. The rate of withering is determined largely by the temperature and humidity of the air. This withering process can be controlled by varying its duration and the thickness of spread of the leaf. Irrespective of the thickness of spread there is always an initial rise in peroxidase. Eventually a maximum peroxidase activity is reached, after which it falls again, this fall being most marked with very thin spreads. Results are quoted in Table V giving the variations in peroxidase activity of leaf withered for 18 and 42 hr. at different thicknesses of spread.

Table V

Lb. of leaf per sq. yard	Peroxidase content		Moisture content	
	18 hr.	42 hr.	18 hr. %	42 hr. %
3	665	237	59.56	24.13
1½	733	379	66.82	51.84
3	784	536	70.26	60.46

The original green leaf contained 570 I.U. per g. dry weight and had a moisture content of 74.30 %.

In experiments in which the rise in peroxidase was recorded at frequent intervals it was found that the rise was most rapid at the commencement of withering. Data of one such experiment, typical of several, are recorded in Table VI, and it will be seen that with a spread of 1 lb. per sq. yard the maximum activity is attained in 6 hr., while with a 3 lb. spread this maximum was not reached for 20-25 hr.

The peroxidase content begins to fall in both cases when the moisture content falls below 72 %. This latter figure must not be taken as a precise one, but there seems no doubt that the rise in peroxidase during withering is checked and then reversed when the moisture falls below a certain critical value.

To summarize these findings, loss of moisture up to a certain point accelerates the increase of peroxidase which takes place in leaf plucked from the bush, but



Table VI

Hours withering	1 lb. spread per sq. yard		3 lb. spread per sq. yard	
	Peroxidase	Moisture	Peroxidase	Moisture
0	423	79.37	423	79.37
2	480	77.54	552	78.52
4	548	77.14	692	78.45
6	618	76.25	716	77.75
8	578	75.54	—	—
12	585	72.56	—	—
15	591	72.26	—	—
24	472	66.95	781	75.34
30	—	—	724	71.80
51	—	—	650	70.70

this increase is checked by too rapid a loss of moisture. The maximum increases are observed when the loss of moisture is restrained by thick spreading of the leaf.

*Effect of the increase in peroxidase during withering on the rate of fermentation*

Were it not for the fact, already commented upon in this paper, that marked variations in the peroxidase activity of green leaf are generally without effect on the fermentation rate, it might be expected that withered leaf would undergo

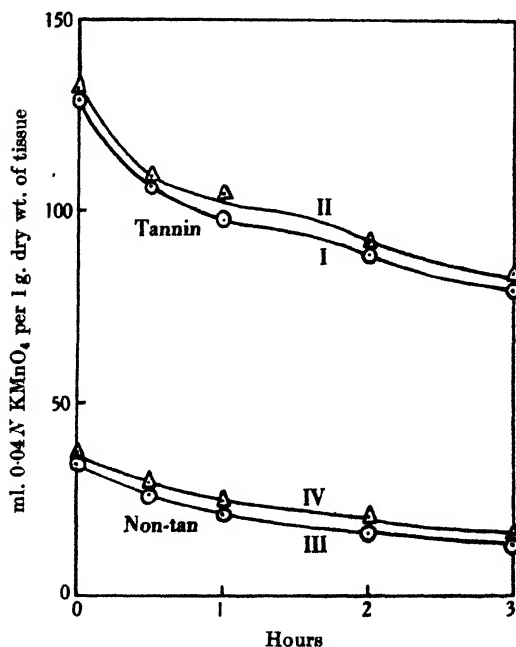


Fig. 2. Tannin and non-tan titres during fermentation. I and III, fresh leaf; II and IV, withered leaf.

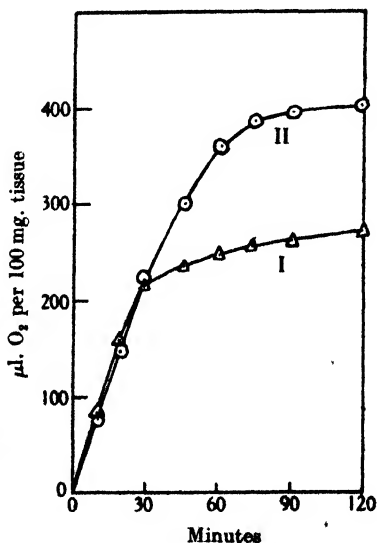


Fig. 3. I, Normal fermentation; II, Do. + 10 mg. tea tannin.

fermentation at a more rapid rate than green leaf. Determinations of the rate of fermentation in fresh and withered leaf were carried out both by measuring the rate of O<sub>2</sub> uptake and by the rate of decrease of the tannin titre. Typical results are illustrated in the accompanying curves (Figs. 2, 3). Details of the

manometric method will be given in Part III. In the tannin titration method a bulk of leaf was separated into two portions. One portion was spread to wither and the other passed through a mincing machine. Two 20 g. portions were at once infused with 500 ml. water and subsequently two such samples were taken and infused at intervals over a 4 hr. period. Tannins and non-tans (i.e. matter oxidized by  $\text{KMnO}_4$  but not precipitated by gelatin and acid-salt) were determined in each of these infusions. The following day, after a 20 hr. wither in which the moisture fell from 75.40 to 58.80 %, a similar series of determinations was made after mincing the withered leaf.

There is no significant difference between the rates of  $\text{O}_2$  uptake or of tannin oxidation in green and withered leaf. In both cases there is a slight separation between the two curves for green and withered leaf. This is not significant but might nevertheless be true, as during withering there is a loss of soluble matter by respiration which may amount to as much as 5 % of the total solid material of the leaf. This loss increases the amounts of other constituents of the leaf when they are expressed on a percentage basis.

During withering a certain amount of protein breakdown takes place. Protein-N in the leaf is determined by complete extraction of the non-protein nitrogenous matter with hot 85 % alcohol, followed by a Kjeldahl estimation of the residual N. This residual N is considered to be protein-N and decreases progressively throughout withering as shown in Table VII.

Table VII

Hours of wither 1½ lb. spread per sq. yard	Protein-N % on dry matter	Moisture content %
—	2.38 ± 0.02	74.30
18	2.19 ± 0.02	66.82
42	1.88 ± 0.05	51.84

The figures given for protein N represent the mean of six 10 g. samples of leaf.

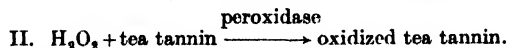
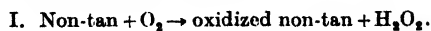
As the decrease in protein-N is unaccompanied by any change in the rate of fermentation, the theory of Kursanov [1935] that protein degradation products protect peroxidase against the inactivating effect of tea tannin is effectively disproved.

#### DISCUSSION

It was concluded in the previous communication that  $\text{H}_2\text{O}_2$ , and not an organic peroxide, is concerned in the oxidation of tea tannin by peroxidase. In the results presented here every line of evidence points to the rate of oxidation of tea tannin being completely independent of the peroxidase content between very wide limits. It must therefore be concluded that the peroxidase-catalysed oxidation of tea tannin in fermentation is preceded by a relatively much slower reaction, in which  $\text{H}_2\text{O}_2$  is formed. Oxidation of tea tannin cannot proceed at a faster rate than the rate of formation of  $\text{H}_2\text{O}_2$ , so that, as long as this latter reaction is unaffected, wide variations in peroxidase activity are without effect on the rate of oxidation of tea tannin.

$\text{H}_2\text{O}_2$  in respiring tissue is a normal by-product of aerobic dehydrogenations in which oxygen functions as the H-acceptor. During fermentation the non-tans undergo oxidation as well as tea tannin and it seems reasonable to suppose that the  $\text{H}_2\text{O}_2$  required for tannin oxidation originates in this oxidation of non-tans. Since 1 mol. of  $\text{H}_2\text{O}_2$  is needed to oxidize 1 mol. of tannin these non-tans should be present in at least equivalent proportions to the tannin, and further there should be approximate equivalence between the amount of tannin oxidized and

the extent of non-tan oxidation. Exact equivalence cannot be expected as the primary oxidation product of tannin may undergo further changes. These expectations are realized. The non-tans account for a substantial proportion of the Lowenthal total  $\text{KMnO}_4$  titre, and frequently in the initial stages of fermentation the decreases in the tannin and non-tan figures are approximately equivalent. There is thus very strong evidence in favour of the following mechanism for the fermentation process.



The nature of these changes will be dealt with in Part III below.

### SUMMARY

There is a marked seasonal variation in the peroxidase content of tea leaf, plucked at weekly intervals, from the same plot. The curve falls into three well-defined periods, each of which is associated with a particular type of manufactured tea.

Generally there is a complete lack of correlation between peroxidase activity and rate of oxidation of tea tannin in the fermentation process. It is concluded that the amount of peroxidase in the leaf is in excess of requirements. The controlling factor is the rate of production of  $\text{H}_2\text{O}_2$  which is formed by the aerobic dehydrogenation of the non-tan oxidizable matter.

The increase in peroxidase content during the withering process is accelerated by loss of moisture from the leaf. Once the moisture falls below a critical level (72 %) peroxidase activity again declines. Protein-N decreases during withering and, as bruised withered leaf undergoes fermentation (tannin oxidation) at the same rate as bruised fresh leaf, the increase in the amount of protein degradation products has no protective effect against the inactivation of the enzymes by tea tannin.

### III. THE MECHANISM OF FERMENTATION

The probable role of the non-tans in fermentation as the source of the  $\text{H}_2\text{O}_2$  required for tea tannin oxidation has been pointed out above. Ascorbic acid suggests itself as the most likely substance to undergo such oxidation in view of the suggestions made by Huszák [1937] and Szent-Györgyi [1937] as to the respiratory mechanism of peroxidase plants generally. Ascorbic acid need not account for the whole of the non-tan titre as the dehydroascorbic acid formed by its oxidation will act as a H-acceptor in the oxidation of other metabolites, ascorbic acid itself being regenerated in this latter reaction. One molecule of ascorbic acid can in this way account for the oxidation of many molecules of such metabolites and there will be a large decrease in the amount of reducing substances (non-tans) with quite small amounts of ascorbic acid present.

Two lines of investigation are suggested by this hypothesis, the elucidation of the nature of the non-tans and the changes they undergo, and an enquiry into the role of ascorbic acid and ascorbic acid oxidase in the fermentation process.

The complete theory of the fermentation process must not only account for the chemical changes observed in fermentation but must also account for such

differences in the rate of fermentation as are observed with Betjan and Kharikatia leaf. Advantage was taken of this marked difference, experiments being made to decide the factor or factors responsible for this difference, in an attempt to gain some more insight into the nature of tea fermentation before proceeding with the lines of investigation just mentioned.

*A comparison of the rates of fermentation in Betjan and Kharikatia leaf*

Neither peroxidase nor tannin contents of Betjan and Kharikatia leaf are sufficiently different to account for any variation in the rate of fermentation. A greater toughness of Kharikatia leaf making it more resistant to the bruising effect of the rollers is also excluded, as with finely minced leaf where damage is more extensive the differences between the two persist as shown in Fig. 4.

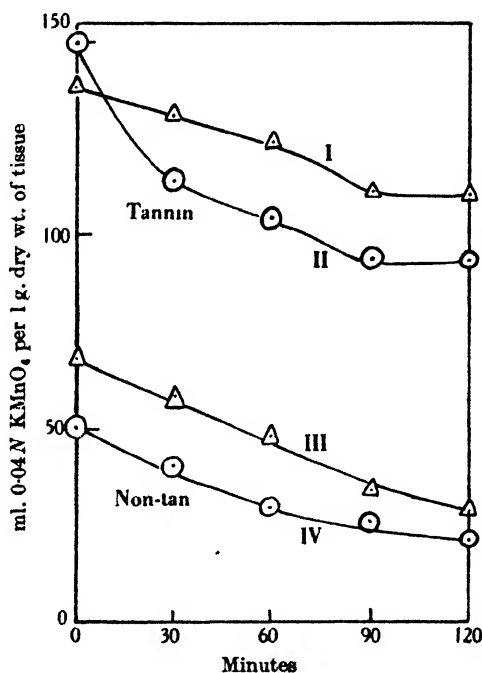


Fig. 4. Tannin and non-tan titres during fermentation. I and III, Kharikatia; II and IV, Betjan.

Any possibility of a peroxidase inhibitor in the Kharikatia leaf has been excluded. Peroxidase inactivation is greater during the fermentation of Betjan leaf than it is with Kharikatia, owing probably to the greater enzymic-inhibiting powers of oxidized tea tannin. Further, a Betjan peroxidase preparation together with H<sub>2</sub>O<sub>2</sub> oxidizes a green leaf infusion obtained from Kharikatia leaf at the same rate as an infusion of the same strength from Betjan leaf. Thus under conditions where H<sub>2</sub>O<sub>2</sub> is introduced into both systems at the same rate the rate of fermentation is the same. It appears therefore highly probable that the difference in fermentation rate is due to a difference in the rate of production of H<sub>2</sub>O<sub>2</sub> in the leaf. If the hypothesis is correct, that the production of H<sub>2</sub>O<sub>2</sub> is due to an enzymic oxidation of ascorbic acid, then the differences between

Betjan and Kharikatia are understandable if the latter is deficient in ascorbic acid oxidase.

There is a certain amount of indirect evidence in favour of this hypothesis. The peroxidase preparation of Roberts & Sarma [1938] from tea leaf is almost without action on a green leaf infusion unless  $H_2O_2$  be first added. In this enzyme preparation the green leaf has been exhaustively extracted with alcohol to free it from tannin, a procedure known to inactivate ascorbic acid oxidase [Srinivasan, 1935]. If the peroxidase be prepared from finely ground up tea leaf by the method of Manskaya [1935], in which tannin is adsorbed by hide powder, the enzyme preparation can oxidize a green leaf infusion directly without the addition of  $H_2O_2$ . While not establishing that the factor producing  $H_2O_2$  is ascorbic acid oxidase, this observation does indicate its possibility, and definitely establishes the presence of a non-tan oxidase. An enzyme preparation obtained in this way with hide powder from Kharikatia leaf oxidizes a green leaf infusion directly without the addition of  $H_2O_2$ , but at a significantly lower rate than a similar preparation from Betjan leaf.

#### *Manometric investigation of fermentation rate*

##### *Experimental technique.*

In determining the rates of  $O_2$  uptake of fermenting tea leaf by the Barcroft-Warburg method it is found most convenient to take 200 mg. portions of green leaf or 150 mg. portions of withered leaf. Such small samples of ordinary rolled leaf would be very unrepresentative. However, if samples of 40–50 g. are minced as finely and rapidly as possible, and the resulting mince well mixed, thoroughly representative samples of even less than 200 mg. can be taken.  $O_2$  cannot diffuse into this fine mince, the surface layer only undergoing fermentation.  $O_2$  uptake is however quite rapid if the leaf is suspended in 3 ml. water and the vessels are shaken at a speed of 90–120 oscillations per min. Until shaking starts  $O_2$  diffuses very slowly into the leaf, so that during preparation of the vessels and attainment of temperature equilibrium comparatively little oxidation takes place. Unless otherwise stated, all experiments are carried out at  $35.5^\circ$ . Lower thermostat temperatures than this cannot be maintained during the Monsoon months. Uptakes are recorded in  $\mu$ l. per 100 mg. tissue wet weight. Agreement between replicates is very good, the standard error of the mean with eight replications varying from 2 to 3 % of the recorded uptakes. Normally all experiments are carried out in triplicate or quadruplicate, when differences of 10 % or more are significant. The leaf itself contains sufficient buffering material to protect the system from any effects due to changes in pH. Comparable amounts of minced leaf were suspended in water alone and in buffers of pH 4.6, 5.6 and 6.6 respectively. In all four cases the rate of  $O_2$  uptake was the same.

#### *The completion of tannin oxidation in fermentation*

$O_2$  uptake is initially quite rapid,  $Q_{O_2}$  may be as high as  $-20.0$ , but after  $\frac{1}{2}$  hr. shaking the rate slows down considerably and after 1 hr. the uptake has come almost to a standstill. If fresh enzyme be added (the hide-powder peroxidase preparation containing "non-tan" oxidase) in amounts roughly equivalent to that originally present in the fermenting leaf, no further  $O_2$  uptake is stimulated. On the other hand, as shown in Fig. 5, after addition of fresh substrate (a green leaf infusion), containing the same amount of tea tannin as was contained in the original portion of green leaf, there is a rapid  $O_2$  uptake. The falling off in the rate of  $O_2$  uptake is therefore due to the exhaustion of substrates. Fig. 6 shows

the greater uptakes recorded when 10 mg. of a tea-tannin preparation (86.4 % pure) is added initially to the fermenting leaf. Fermentation does not come to a standstill till much later in this case, from which it can be concluded that the slowing down in the fermentation rate is due to the completion of the oxidation of the tannins. From the slope of the curve in Fig. 5 after the addition of fresh substrate it can be calculated that the enzymes in the fermented leaf retain about 80 % of their activity.

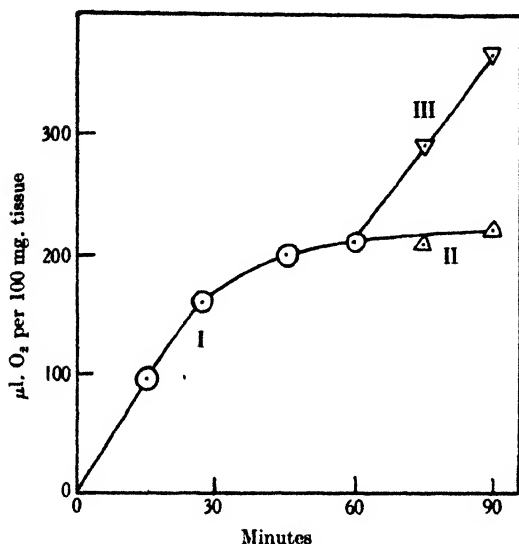


Fig. 5. I, normal fermentation; II, fresh enzyme added; III, fresh substrate added.

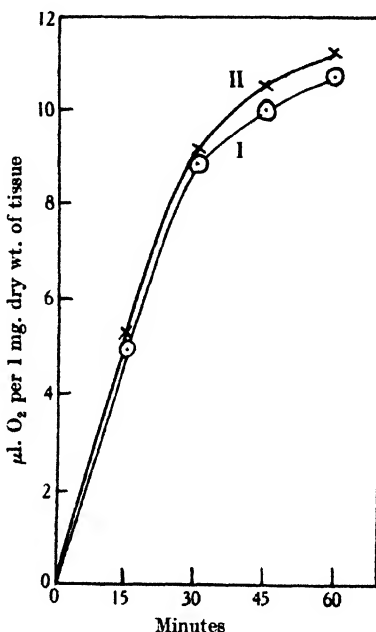


Fig. 6. O<sub>2</sub> uptakes during fermentation. I, fresh leaf; II, withered leaf.

### Production of CO<sub>2</sub>

During fermentation a certain amount of CO<sub>2</sub> is produced as is shown by the lower net uptakes when no KOH is added to the central cups in the Warburg vessels. Very little CO<sub>2</sub> is retained by the suspension of fermenting leaf, so that the differences between the recorded uptakes with and without KOH gives an approximate figure for the CO<sub>2</sub> evolved if allowance is made for the differences in the constants of the vessels for O<sub>2</sub> and CO<sub>2</sub>.

The CO<sub>2</sub> produced in the respiration of normal leaf is largely a product of carbohydrate oxidation, and it is not unreasonable to assume that in fermenting tea leaf it has a similar origin. The R.Q. of carbohydrate oxidation to CO<sub>2</sub> is 1.0, so that an estimate of the O<sub>2</sub> utilized in tannin oxidation is afforded by subtracting the CO<sub>2</sub> figures from the total O<sub>2</sub> uptake. Knowing the original amount of tea tannin in the tea leaf from its Löwenthal titre (1 ml. *N* KMnO<sub>4</sub> = 0.0416 g. tea tannin) and assuming that Tsujimura's [1930; 1931, 1, 2] structure of tea tannin, corresponding with a mol. wt. of 442 is correct, the number of atoms of O required by each molecule of tannin can be calculated from the O<sub>2</sub> consumed in tannin oxidation. To quote one case, one sample of leaf had a tannin titre of 130.4 ml. 0.04 *N* KMnO<sub>4</sub> per g. dry weight, and the total O<sub>2</sub> uptake recorded per g. dry weight in 1 hr. after subtracting the value for carbohydrate oxidation was

5584  $\mu$ l. If the tea tannin requires 1 atom of O per molecule the uptake would be 5498  $\mu$ l., so that it can be concluded that in this particular case 1 atom of O only is required for complete oxidation. The average of six such calculations gives  $1.08 \pm 0.08$  O which does not differ significantly from unity.

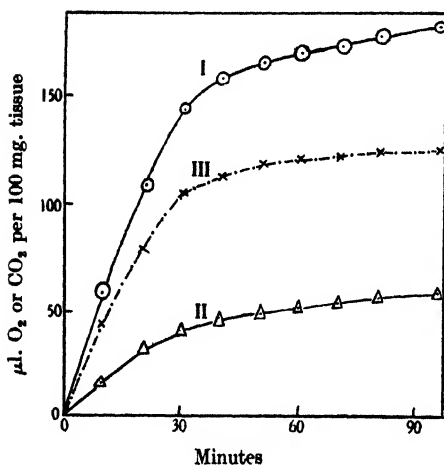


Fig. 7. I, total O<sub>2</sub> uptake; II, CO<sub>2</sub> output; III, O<sub>2</sub> consumed in tannin oxidation.

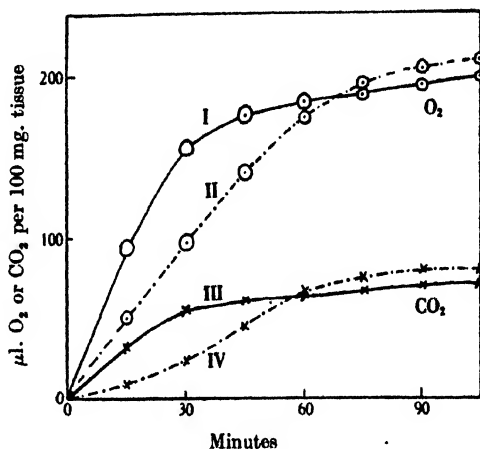


Fig. 8. I and III, Betjan; II and IV, Kharikatia.

There is always a marked parallelism between O<sub>2</sub> uptake and CO<sub>2</sub> output as is shown in Figs. 7 and 8. In the latter figure which gives the O<sub>2</sub> uptakes and CO<sub>2</sub> outputs of fermenting Betjan and Kharikatia leaf it will be seen that, with the slower-fermenting leaf, tannin takes longer to be completely oxidized and the rate of carbohydrate oxidation takes a correspondingly longer time to fall off. The ratios of the rates of tannin and carbohydrate oxidation are approximately the same for both types of leaf. This connexion between the rates of carbohydrate and tannin oxidation will have to find some explanation in any mechanism suggested for the fermentation process.

#### *Identification of glucose as fermentable non-tan*

The production of CO<sub>2</sub> in amounts very nearly equivalent to half the total O<sub>2</sub> uptake during fermentation suggests that the non-tans which are oxidized during fermentation are carbohydrates. The decrease in the KMnO<sub>4</sub> titre of non-tans and the amount of CO<sub>2</sub> produced in fermentation are approximately equivalent, as is shown by the equality of the ratios

$$\frac{\text{CO}_2 \text{ output}}{\text{O}_2 \text{ uptake}} = \frac{\text{Decrease in non-tan titre (in ml. KMnO}_4\text{)}}{\text{Decrease in tannin + non-tan titre}}$$

both of which are about 0.4 at the end of fermentation.

The majority of the non-tan oxidizable matter may be extracted from green leaf in the following manner, advantage being taken of the precipitation of tannins by lead acetate. The leaf is extracted with 80% alcohol and an aliquot of the extract representing 20 g. of green tissue concentrated on the water bath to 5–10 ml. 50 ml. of water are added and the solution washed into a 250 ml. flask. The extract is cleared by the addition of 3 ml. saturated neutral lead acetate after which it is made up to 250 ml. The solution is then filtered, de-leaded with H<sub>2</sub>S and again filtered. Aliquots of the clear filtrate are freed from H<sub>2</sub>S

and are used for the determination of reducing sugars directly by the Shaffer-Hartmann [1925] method, results being expressed in mg. of glucose. Such an extract contains most of the oxidizable and all of the fermentable non-tan matter in green leaf, that is to say the whole of the non-tans oxidized during fermentation are present in this extract.

During fermentation there is a fall in the reducing sugar content of the leaf. Fresh green leaf in one experiment contained 3.12% reducing sugar as glucose while the same leaf after fermentation contained only 1.40% glucose. Both figures are expressed on a dry weight basis. Further evidence for the carbohydrate nature of the non-tan fermentable matter is its fermentation by yeast.  $\text{CO}_2$  is produced at the same rate both from this extract and from a glucose solution of comparable strength. The reducing substance in the extract has been identified as glucose by the isolation in high yield of its osazone. It may therefore be concluded that the non-tan oxidizable matter which is oxidized in the fermentation of tea is glucose.

#### *Ascorbic acid and tea fermentation*

Tea tannin which seriously interferes with methods of ascorbic acid determination may be removed by addition of neutral lead acetate to a green leaf infusion. The ascorbic acid in the filtrate may then be determined by the method of Stevens [1938] in which 20 ml. of the solution are titrated with  $N/100 \text{ I}_2$  after the addition of 4 ml.  $12N \text{ H}_2\text{SO}_4$ . Such estimations of the ascorbic acid content of green leaf indicate that 1 g. of fresh green leaf contains about 1 mg. ascorbic acid, but this must be regarded as a preliminary figure only. If excess ascorbic acid be added to the green leaf infusion the whole of this excess is found in the lead acetate filtrate, so that the above method can be employed to determine changes in ascorbic acid added to tea juice.

20 ml. of expressed tea leaf juice were mixed with 20 ml. of a 0.44% solution of ascorbic acid and diluted to 100 ml. A further 20 ml. portion of juice was diluted with water and boiled for 5 min. after which 20 ml. of the ascorbic acid solution were added and the volume made up to 100 ml. Both mixtures were incubated for 1 hr. at room temperature ( $30^\circ$ ) with frequent shaking. Tea tannin was then removed by the addition of 5 ml. saturated lead acetate and ascorbic acid determined iodimetrically in 20 ml. portions of both solutions, and in a control portion of the original ascorbic acid solution treated in the same way with lead acetate.

Table VIII

	ml. $N/100 \text{ I}_2$
Control	19.1
Ascorbic acid + boiled tea juice	12.7
Ascorbic acid + unboiled tea juice	2.9

The unboiled tea juice brings about a much greater oxidation of ascorbic acid, which is evidence for the presence of a thermolabile catalyst of ascorbic acid oxidation in the tea leaf.

Addition of excess ascorbic acid to fermenting leaf has no significant effect on the rate of  $\text{O}_2$  uptake, as is shown in Fig. 9. This rate however is maintained for a longer period and the total uptake is greater by an amount approximately equivalent to the ascorbic acid added. 1.60 mg. of ascorbic acid were added for each 100 mg. of fermenting leaf. This quantity of ascorbic acid requires  $102 \mu\text{l. O}_2$  for complete oxidation and the increase recorded is  $90 \mu\text{l.}$  which is in sufficiently close agreement.



For some 20–25 min. no  $\text{CO}_2$  is produced whatever, indicating that no carbohydrate breakdown is taking place. During the same period the leaf remains bright green and there is no significant fall in its tannin titre. It was shown in the previous communication that such concentrations of ascorbic acid completely

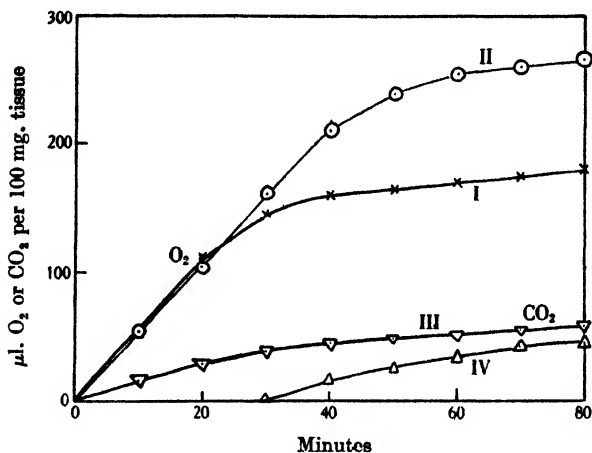
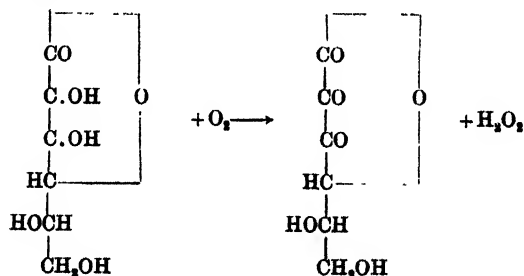


Fig. 9. I and III, normal fermentation; II and IV, Do. + excess ascorbic acid.

inhibit peroxidase activity, so that it must be concluded that during this initial period of 20 min. the ascorbic acid inhibits both tannin and carbohydrate oxidation. The  $\text{O}_2$  uptake during this period is 105  $\mu\text{l.}$ , just equivalent to the excess ascorbic acid added, and one is justified in concluding that when such excess of ascorbic acid is present it is oxidized away, after which tannin and carbohydrate oxidation can take place as usual. During this period of ascorbic acid oxidation the rate of  $\text{O}_2$  uptake is exactly the same as it is in normal fermentation. From this it can be concluded that the rate we are measuring in all manometric experiments on fermentation is that of the enzymic oxidation of ascorbic acid. The same experiment provides conclusive evidence of the role of ascorbic acid in fermentation and confirms the earlier conclusion that the rate of fermentation would prove to be the same as the rate of production of  $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  is of course produced in the aerobic oxidation of ascorbic acid.



The hypothesis that Kharikatia leaf contains less ascorbic acid oxidase than Betjan can now be tested. As shown in Fig. 8, Kharikatia has a lower rate of  $\text{O}_2$  uptake and hence it produces  $\text{H}_2\text{O}_2$  at a lower rate. If the fermenting leaf from both "jats" be saturated with ascorbic acid (1.76 mg. per 100 mg. leaf) these differences in the rate of  $\text{O}_2$  uptake should persist. The initial rates of  $\text{O}_2$  uptake,

where the only reaction taking place is ascorbic acid oxidation, are quite different in the two cases, as shown in Fig. 10. As the substrate concentration is the same in both cases it must be concluded that the difference must lie in their ascorbic acid oxidase contents.

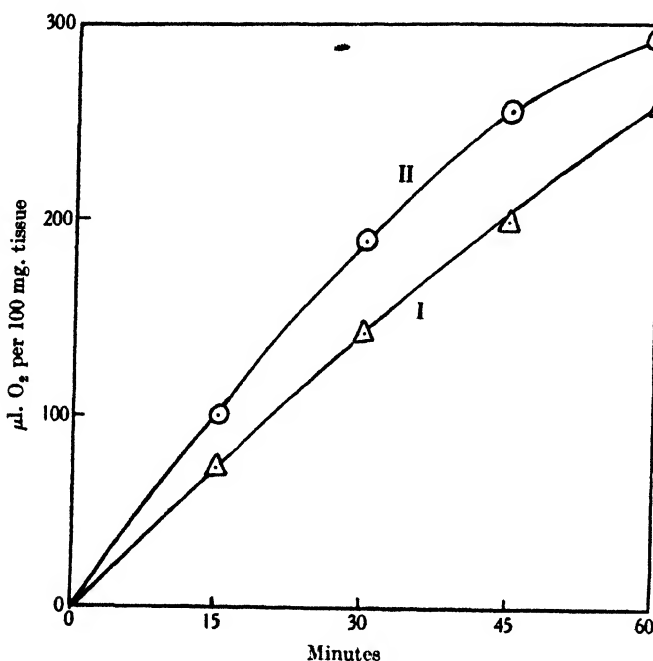


Fig. 10. I, Kharikatia + excess ascorbic acid; II, Betjan + excess ascorbic acid.

#### *Variation of fermentation rate with amount of leaf*

Normally when measuring the rate of  $O_2$  uptake of respiring tissues the rate per unit weight of tissue is independent of the amount of tissue taken and of the volume of liquid in which the tissue is suspended as the reactions studied are surface reactions and not homogeneous.

In the particular case under consideration here, however, such variations have a significant effect on the rate of  $O_2$  uptake. The smaller the amount of leaf taken the more rapid is the rate of the reaction per unit weight of tissue. It is therefore important to ensure that the same amount of leaf is taken for every experiment. Variations of 10 mg. in the amount of leaf weighed out are permissible, but greater deviations than this produce significant variations in the  $O_2$  uptake.

Table IX gives the  $O_2$  uptakes per 100 mg. leaf when quantities of 100 and 250 mg. of leaf undergo fermentation.

Table IX

Wt. of leaf mg.	15 min.	30 min.	45 min.	60 min.
250	99	182	204	213
100	132	190	204	209

Further investigation of this effect shows that, with smaller amounts of tissue, while tannin oxidation proceeds relatively faster carbohydrate oxidation is slowed down.

Table X

Wt. of leaf mg.		15 min.	30 min.	45 min.	60 min.
100	Tannin	84	110	107	111
	Carbohydrate	23	35	44	51
200	Tannin	60	102	117	122
	Carbohydrate	33	55	59	63

The figures give the  $O_2$  uptakes in  $\mu$ l. per 100 mg. fresh green leaf for tannin and carbohydrate oxidation respectively.

### DISCUSSION

There seems to be no doubt that the first stage in the fermentation of tea is the enzymic oxidation of ascorbic acid, with the formation of dehydroascorbic acid and  $H_2O_2$ . Peroxidase and  $H_2O_2$  then oxidize the tea tannin. As each molecule of tea tannin takes up 1 atom of O only in its oxidation, and tea tannin contains the catechol grouping, the primary product of oxidation of tea tannin is most probably an *o*-quinone.

There is ample evidence that the primary product of oxidation undergoes irreversible condensations to form a series of products whose colours range from bright reddish-brown to dark brown. This subject will form the basis of a further communication at a later date. Meanwhile it can be taken that the *o*-quinone from tea tannin can be removed from the sphere of action by condensation.

The dehydroascorbic acid is an effective H-acceptor and will function as such in the dehydrogenations taking place during the oxidative breakdown of carbohydrates, the ascorbic acid being reformed in the process. A continuous regeneration of ascorbic acid must necessarily take place as about 20 mol. of tea tannin are oxidized for every mol. of ascorbic acid present in the leaf.

This scheme however fails to account for all the observed facts. There is no reason why carbohydrate oxidation should slow down when tannin oxidation has come to a standstill as the catalase in the leaf could deal with the  $H_2O_2$  produced in the first stage of the process and prevent its rising to toxic concentrations. Further, all the reactions involved are enzymic and are thus heterogeneous, so that no explanation of the variations in fermentation rate with the amount of leaf taken can be advanced.

The simple scheme outlined above takes no account of the possible interaction between ascorbic acid and the primary oxidation product of tea tannin.

Ascorbic acid + *o*-quinone  $\rightarrow$  dehydroascorbic acid + catechol.

When this is considered it will be seen that two possible fates await the *o*-quinone on its formation. It can undergo an irreversible condensation or it can be reduced again to tea tannin by ascorbic acid.

The concentration at any moment of dehydroascorbic acid, on which the rate of carbohydrate oxidation depends, will therefore be determined partly by the concentration of the *o*-quinone. When tannin oxidation approaches completion the concentration of *o*-quinone will fall and with it the rate of oxidation of carbohydrate. The parallelism between tannin and carbohydrate oxidation can therefore be deduced as a necessary consequence of this reaction between ascorbic acid and the *o*-quinone.

This reaction is both homogeneous and bimolecular. If less than the normal weight of tissue be suspended in the normal volume of water in the Warburg

vessels the mass action effect will be to increase the relative concentration of the *o*-quinone and to decrease that of the dehydroascorbic acid. The net result will be to accelerate the complete transformation of tea tannin into its oxidized and condensed products and to decrease the rate of carbohydrate oxidation.

We may therefore write down the complete reaction scheme for fermentation as follows.

- (1) Ascorbic acid +  $O_2$   $\xrightarrow{\text{ascorbic acid oxidase}}$  dehydroascorbic acid +  $H_2O_2$ .
- (2)  $H_2O_2$  + tea tannin  $\xrightarrow{\text{peroxidase}}$  tannin *o*-quinone.
- (3) *o*-Quinone  $\rightarrow$  condensation products.
- (4) *o*-Quinone + ascorbic acid  $\rightarrow$  tea tannin + dehydroascorbic acid.
- (5) Dehydroascorbic acid + glucose  $\xrightarrow{\text{zymase}}$   $CO_2$  + ascorbic acid.

The "fermentation" of tea, like the browning of various other plant tissues on injury, is a case of decompensated respiration, and it should be possible to arrive at the mechanism of the true respiratory process in the tea leaf from a consideration of the "fermentation".

Respiration differs from "fermentation" in that the net change in the respiration process is the oxidation of carbohydrates to  $CO_2$ . No permanent changes in the tannins or other catechols take place.

If tea tannin can participate in normal respiration some explanation of its stability under these conditions must be found. While the R.Q. of respiring leaf is 1.0 that of fermenting leaf is initially about 0.3. Animal tissues show a similar fall in R.Q. after extensive damage. Thus the R.Q. of liver slices is 0.79, but finely minced ox-liver has an R.Q. of 0.37 as shown by Roberts [1936]. Further, the  $O_2$  uptake of this finely minced liver was shown to be due almost entirely to purine base oxidation by xanthine oxidase. Unlike most dehydrogenases this enzyme can utilize molecular  $O_2$  without the aid of a coenzyme, so that it would appear that the predominance of purine base oxidation in minced liver is due to a dispersal of coenzymes in the mincing process, leaving xanthine oxidase alone, with its full activity. A similar dispersion of coenzymes would be expected to follow the extensive damage done to tea leaf in the rollers or on mincing. The result of such a dispersion would be a slowing down of the dehydrogenations in which dehydroascorbic acid functions as the H-acceptor, and a consequent accumulation of dehydroascorbic acid in the system. This in its turn would involve a lower concentration of ascorbic acid and consequently a slower reduction of the *o*-quinone to tea tannin. If the *o*-quinone is not reduced as soon as it is formed it can then undergo further irreversible changes into condensation products and this process will continue until the whole of the tannin has been removed from the system.

Under this scheme respiration and fermentation differ only in the velocity of dehydrogenations taking place in carbohydrate breakdown.

The difficulty which stands in the way of accepting tea tannin as an  $O_2$  carrier in normal respiration is its high concentration, accounting as it does for about 20% of the total solid matter in the green leaf. Quercitrin also occurs in the tea leaf and in concentrations far more like those of  $O_2$  carriers. In this case quercitrin would be the catechol compound oxidized by peroxidase in normal respiration, and tannin would be involved in the reactions only after damage to the tissue had permitted it to mingle with the other constituents of the respiration cycle.

It is at the moment impossible to decide between these two hypotheses but some observations of Mr C. J. Harrison favour the former. If green leaf is exposed to  $\text{CHCl}_3$  vapour the leaf reddens and takes up  $\text{O}_2$  very rapidly. This observation is readily explained by a greater sensitivity of dehydrogenases to the toxic effect of  $\text{CHCl}_3$ . The consequent partial inhibition of carbohydrate oxidation would be expected on the above theory to lead to the production of condensation products of tannin and this is in fact observed. If green leaf is heated to  $100^\circ$  all the enzymes are inactivated and the leaf remains green. If, on the other hand, leaf is kept at  $40\text{--}50^\circ$  it reddens. This reddening can be accounted for in the same way as the reddening after exposure to  $\text{CHCl}_3$  vapour. The dehydrogenases are more susceptible to the inactivating effect of heat than the other enzymes concerned in respiration, so that moderately high temperatures, by a greater inhibition of carbohydrate oxidation, might be expected to cause some decompensation in respiration with a resulting formation of tannin condensation products.

#### SUMMARY

A complete reaction scheme for the fermentation process is deduced from the experimental data available. The  $\text{H}_2\text{O}_2$  necessary for the oxidation of tea tannin by peroxidase originates in the aerobic oxidation of ascorbic acid, and this reaction controls the rate of the whole process. A shortage of ascorbic acid oxidase, as in the case of the Kharikatia leaf, results in a slower rate of fermentation.

The oxidizable non-tan which decreases during fermentation is identified with glucose. Dehydroascorbic acid functions as the H-acceptor in the oxidative breakdown of the latter.

One atom of O only is taken up per molecule of tea tannin during fermentation. The oxidation product then undergoes an irreversible change into condensation products.

The relation of the fermentation process to normal respiration is discussed.

The author wishes to express his thanks to Mr P. H. Carpenter, Chief Scientific Officer, and the other officers on the Station for much useful criticism and advice during the course of this work, and to the Indian Tea Association for permission to publish these results. Thanks are also due to Mr S. N. Sarma and Mr P. B. Sen Gupta for their skilful assistance.

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#### CORRECTION

In the first paper of this series *Biochem. J.* **32**, 1821, line 41, delete "infusion".

# CV. THE NUTRITIVE VALUE OF WHEATEN PRODUCTS

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*(Received 23 March 1939)*

WHEATEN products used as flour and bread are generally held to be of low nutritive value apart from their capacity to supply energy. It is certainly true that rats cannot be kept in good health or even alive for any length of time on a diet of bread alone. If the bread is made of white patent flour there is no growth at all and the animals die after 2 months. When the bread is made of 3 parts of white flour and 1 part of germ, constituting a foodstuff rich in the vitamin B complex, growth occurs for a time, but ceases after about 2 months and after another 2 months the animal dies (Fig. 1). This nutritive failure is attributed mainly to the low biological value of the proteins of wheat. We found, however, that rats could live and grow for many months in apparently good health on a diet composed almost entirely of wheaten products, namely, white flour to which sufficient wheat germ had been added to give an adequate supply of the vitamin B complex (3 parts of flour and 1 part of germ) and in which the abnormal Ca/P ratio of 1 : 9 had been corrected by the addition of 3% calcium lactate, to produce a Ca/P ratio of 1 : 0.45.

The average mineral content of a germ-flour mixture is as follows:

	% in moist flour
CaO	0.04
P <sub>2</sub> O <sub>5</sub>	0.65
MgO	0.16
K <sub>2</sub> O	0.28
NaCl	1.53
Fe	0.002

The remarkable improvement in the nutritive value of a germ-white flour mixture brought about by correcting the unfavourable Ca/P ratio by the addition of calcium lactate does not take place when calcium lactate is added to white flour alone. On this latter diet the rats show only a slight initial growth and die after about 2 months with deficiency symptoms just as they do on a diet of white flour alone. This difference is illustrated in Figs. 2 and 3, which represent typical growth curves of individual animals.

The following experiments were carried out in order to study in some detail the nutritive value of a diet composed entirely of wheaten products with a corrected Ca/P ratio. The plan of these experiments was to test the length of survival of male and female rats kept on a germ-white flour-calcium lactate diet and to determine whether it interfered with breeding and with the rearing of the young. In this way it seemed possible to obtain some direct evidence of the nutritive value or defects of the proteins of wheat and of wheaten cereals as a whole.

White Wistar rats bred in the laboratory were selected for these experiments as they were found to give good indications of rickets and grew well on the usual rachitic diet. Coloured rats of unknown origin, which, when fed on the same

rachitic diet did not grow well and in some cases died after 3-4 weeks, did not give equally consistent results. The food mixture was mixed with tap water. In each experiment one male and two female rats were kept together in one cage. Many of the females became pregnant and littered. Of the newly born rats many died; the survivors, after having been weaned, were at once put on the flour germ-calcium lactate diet. When they had reached sexual maturity they were again divided into groups of one male and two females for each box, in

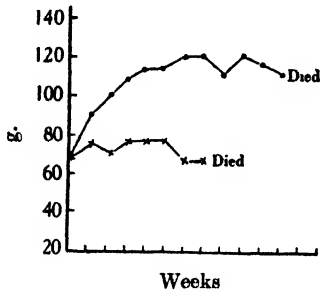


Fig. 1. Germ bread and white bread.  
●—● Germ bread. ×—× White bread.

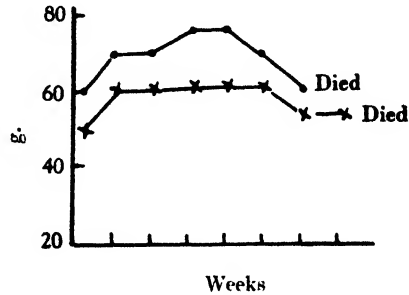


Fig. 3. White flour + NaCl + 3% calcium lactate. ♂ ●—●. ♀ ×—×

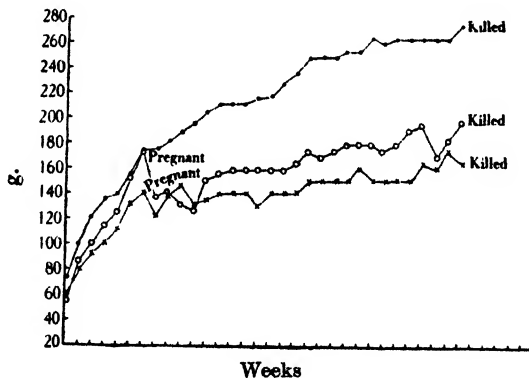


Fig. 2. Germ flour + 3% calcium lactate. ♂ ●—●; ♀ ○—○; ♀ ×—×.

order to see whether breeding from them could be continued on this diet. This was successful in a number of experiments. Thus, in Exp. 512 (Table I) two litters were obtained, of which 10 rats survived weaning and lived for 9 months or more on the cereal diet. A similar result was obtained in Exps. 549, 560 and 561 in which 17 young rats survived weaning. Of these rats, 1 lived for 8 months and 4 for 12 months on the cereal diet. The maximal weights of these 4 rats were 235 and 170 g. for two males, 220 and 160 g. for two females. They lost in weight eventually and were killed. One of the female rats (no. 1819) had 3 litters of 7, 7 and 8 young respectively, all of which, however, died or were eaten by the mother; the other rat (no. 1820) had one litter, all the members of which were eaten by the mother.

The results of the breeding experiments are collected in Table I. It is evident from the experiments that a diet composed entirely of wheaten products, in which the Ca/P ratio is corrected, is of a sufficiently high nutritive value to enable the animals not only to live and to grow, but also to reach sexual maturity,

Table I. *Breeding records*

Exp. no.	Rat no.	No. of young in litter			Remarks
		1	2	3	
374	1204	7	—	—	None survived
380	1228	2	—	—	"
512	1625	5	--	--	5 survived
	1626	7	--	—	5 survived } Survivors grouped to form Exps. 550, 551. No young obtained
549	1731	9	—	—	9 survived
	1732	3	—	—	2 survived
560	1758	4	—	—	None survived
	1759	5	--	—	4 survived } Survivors grouped to form Exp. 580
561	1761	2	--	—	2 survived
	1762	5	--	—	None survived
580	1819	7	7	8	Third generation, none survived
	1820	?	—	—	None survived
653	2043	5	4	—	"
654	2046	7	—	—	"

Table II. *Survival periods of 21 rats*

Months ...	4	5	6	7	8	9	10	11	12	13	24
No. of rats surviving after period shown (all lived at least 4 months)	18	16	15	14	11	8	8	7	2	1	0

to breed and, in some cases, to suckle the young. This second generation can then again be reared on the diet and can breed, but so far we have not succeeded in rearing a third generation on this cereal diet.

Another method for testing the nutritive value of this diet was to determine the period of survival of rats kept on the diet. The results are given in Table II. The maximum age of the albino rat is, according to Donaldson, 3 years. There are apparently no observations on record concerning the mortality of rats on different diets composed of single natural foodstuffs, so that a comparison is not possible. Table II shows that the diet, while not an optimum one, enables all the animals to reach sexual maturity and about 30 % to live for a year or longer. The bones were well formed, as the following analyses show: bone ash in dry, fat-extracted bone, 60.7 %. The bone ash from the bones of a similar rat fed on a normal mixed diet was found to be 56.3 %. None of the rats observed in our experiments developed rickets, xerophthalmia or septic glands. The lungs were normal except that in some of the animals kept on the diet for a year or more, there was a moderate degree of the septic bronchiectasis characteristic of a deficiency of vitamin A. Nor could the cause of death in this series be identified with any specific lesion which could be referred to an inadequacy of the protein or to any other defect of the diet. The causes of death appeared to be various intercurrent diseases. These diseases affected the body-weights, which declined during the last few weeks before death.

In order to search for the presence of any specific lesion which might be obscured by the diseases which killed the animals in the preceding series, a large number of rats were placed on the diet and killed at intervals while apparently still well. Table III contains data concerning the sex, age and increase in weight of the rats observed in this series. No specific lesion was found in these animals. A further experiment to test the survival was carried out with 6 young rats. Of



Table III. *Records of rats killed after varying periods on diet*

Months ...	2	3	4	5	6	7	8	9	10	11	12	13	24
Maximal weights, g.	130	110	190	190	190	210	—	285	200	185	235	255	280
	120	100	145	—	240	240	—	195	150	—	170	205	—
	125	120	165	—	160	210	—	175	—	—	210	205	—
	120	—	130	—	130	—	—	—	—	—	160	—	—
	—	—	160	—	—	—	—	—	—	—	—	—	—
Sex and no.	F 4	F 3	M 1	F 1	M 2	M 3	—	M 1	M 1	M 1	M 2	M 1	M 1
	—	—	F 4	—	F 2	—	—	F 2	F 1	—	F 2	F 2	—

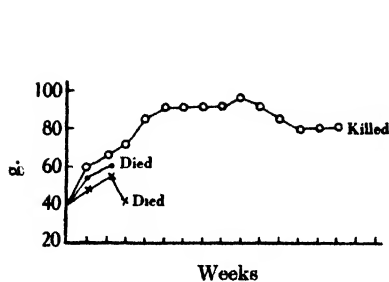


Fig. 4. Raw steak. ♂ —•—; ♀ ×—×; ♀ ○—○.

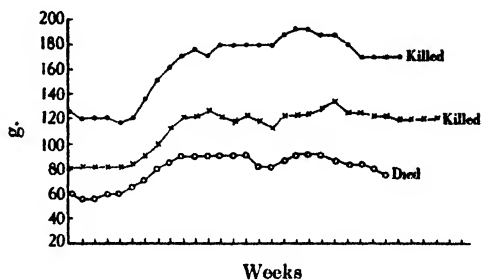


Fig. 5. Raw steak + 5.5% calcium lactate Ca:P::1:0.5. ♂ —•—; ♀ ×—×; ♀ ○—○.

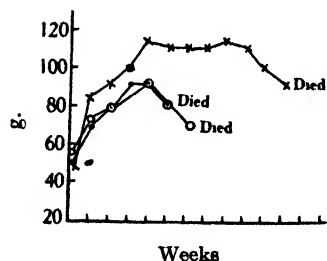


Fig. 6. White bread + 30% raw steak. ♂ —•—; ♀ ×—×; ♀ ○—○.

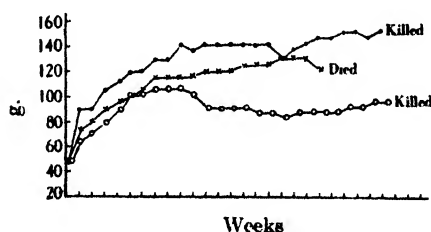


Fig. 7. Germ bread + 30% raw steak. ♂ —•—; ♀ ×—×; ♀ ○—○.

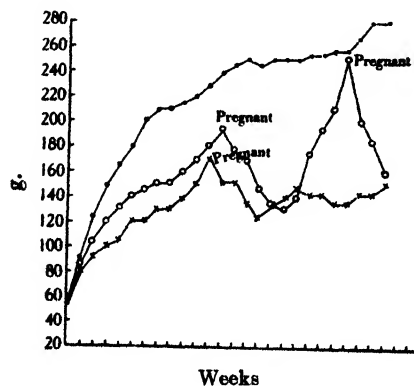


Fig. 8. Germ bread + 30% cheese. ♂ —•—; ♀ ×—×; ♀ ○—○.

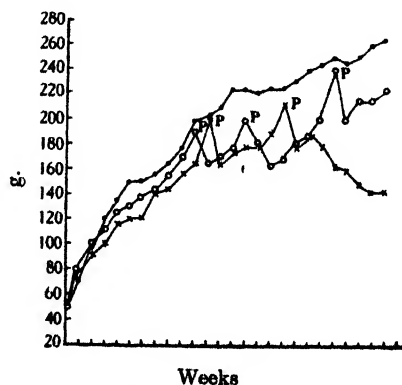


Fig. 9. White bread + 30% cheese. ♂ —•—; ♀ ×—×; ♀ ○—○.

these, 1 died after 4 months, 1 after 5 months, 1 after 10 months, while 2 rats were alive after 11 months.

For comparison, similar experiments were carried out with another important foodstuff, namely meat, the proteins of which are supposed to have a higher biological value than the proteins of wheat. The growth charts show the following results.

Meat alone was unable to maintain growth or even life for more than 5 weeks (Fig. 4). When the Ca/P ratio of meat, which is even below that of bread, having the value 1/20, was corrected by the addition of 5.5% calcium lactate to bring the Ca/P ratio to the value of 1/0.5, there was a temporary period of growth which ceased after 10 weeks. The animals continued to live for 6 months, but lost in weight, looked ill and emaciated and were then killed (Fig. 5). The nutritive value of meat alone, as tested by this method, is therefore inferior to that of the wheaten cereal mixture. On a diet of 2 parts of white bread and 1 part of meat, the animals did almost as badly as on white bread alone (Fig. 6), while the diet of germ bread and meat prolonged life, without, however, allowing of a survival as long as the diet of germ flour and calcium lactate. Growth soon became stationary, the animals declined in weight and their condition deteriorated, so that they had to be killed after 23 weeks (Fig. 7). It should be noted that the Ca/P ratio 1/12 of this diet is below that of bread alone and, therefore, more unfavourable.

On the other hand, a diet of bread and milk, or bread and cheese gave excellent results, even when white bread was used. The rats grew rapidly and produced several litters (Figs. 8 and 9) and after 12 months the animals are still alive and in excellent condition.

#### SUMMARY

On a diet composed entirely of wheat cereals, namely white flour and germ, a vigorous strain of rats will grow progressively, reach sexual maturity, remain in good health for many months and will breed, provided that the unfavourably low Ca/P ratio of this diet has been corrected by the addition of calcium lactate in such an amount as to produce a Ca/P ratio of 1 : 0.5. On such a diet some rats have been kept alive for a year or more. If the Ca/P ratio is not corrected, growth proceeds for a month only and then ceases and all the animals die within 3-4 months.

The addition of calcium chloride or of calcium carbonate does not give such good results, whilst calcium citrate is as effective as calcium lactate.

These results show that the nutritive value of the wheaten cereal mixture used in these experiments is very high. The proteins of wheaten cereals have, therefore, a much higher biological value than has been attributed to them hitherto. The cereal mixture contains a potential supply of vitamin A in the form of carotene, but the amount is not sufficient to afford complete protection when the diet is given over long periods. This is confirmed by figures given by Copping [1939]. Using rats as test objects, the main defect of cereals is the low amount of calcium and probably also of iodine. When this is corrected the mineral content is adequate.

In a diet composed of natural foodstuffs, the main nutritional defects of cereals are corrected by the addition of milk and its products, butter and cheese. Milk and cheese are among the few foodstuffs rich in calcium. They are also rich in vitamin A, and so is butter. Milk and its products represent, therefore, the rational dietetic complement to cereals, while the combination of meat with

cereals is of doubtful nutritive value, since it aggravates the disproportion of calcium and phosphorus. For the human dietary, which requires an extraneous supply of vitamin C, the inclusion of foodstuffs rich in vitamin C—vegetables and fruits—is required.

The experiments were carried out at the suggestion and under the supervision of Dr W. Cramer.

#### REFERENCE

Copping (1939). *Nutrit. Abstr. Rev.* **3**, 563.

# OBITUARY NOTICES

GEORGE BARGER

1878-1939

THE sudden death on 6 January 1939 at the age of 60 years of Prof. George Barger removes one of the most distinguished of the group of chemists and physiologists who were responsible for the formation of our Society and to whose work we owe in large measure the development of Biochemistry in this country.

George Barger was born in Manchester in 1878, his father Gerrit Barger being a Dutch engineer and his mother English. He received his school education in Utrecht where the family settled in his early childhood. At the age of 16 years he chose to return to England to complete his education and entered University College, London, with a scholarship which he won on the University Matriculation Examination: here he commenced his studies for the London B.Sc., later proceeding with a scholarship to King's College, Cambridge. As an undergraduate student Barger was almost equally interested in chemistry and botany, and he was placed in the first class in both of these subjects in Part II of the Natural Sciences Tripos on the last occasion on which more than one subject could be offered for this examination.

After leaving Cambridge Barger was appointed to a demonstratorship under the late Prof. Errera in the department of Botany in the University of Brussels. During the two years which he spent in Brussels he worked at two problems, the chemistry of the glucoside saponarin, and the micro-method of molecular weight determination which bears his name; the latter represents the first of his scientific contributions to find application in the field of biological chemistry and it is significant in indicating his early appreciation of the importance of micro-methods in the development of this branch of science.

In 1903 Barger returned to England and entered the Wellcome Physiological Laboratories as chemist. The decision to take this post, although it was only reached after considerable hesitation, proved to be of the greatest importance for his subsequent career; it brought him into close contact for six years with an active group of biological workers, collaboration with whom was not only fruitful in immediate results but influenced the whole of his later work.

In 1909 Barger returned to academic work, being appointed in that year Head of the Department of Chemistry at Goldsmith's College in the University of London, and in 1913 he became Professor of Chemistry at the Royal Holloway College.

One of the first actions of the Medical Research Committee which was formed in 1914 was to collect its own team of research workers; Barger was appointed to be a member of the group and continued to hold this post during the period of the War.

In 1919 he was appointed to the newly constituted Chair of Chemistry in Relation to Medicine at Edinburgh and he held this position for eighteen years. A little more than a year before his death he became Regius Professor of Chemistry in the University of Glasgow; the latter important appointment was a fitting recognition of the distinguished position which he had attained in the profession of Chemistry in this country; it brought with it heavy burdens, but he threw himself into the arduous task of reorganizing a large department with

conspicuous freshness and enthusiasm; it is sad to think that the work with which he had made so much progress should have had to be left unfinished.

The aspect of Barger's scientific work which will be of major interest to readers of the *Biochemical Journal*, and for which indeed he will perhaps be chiefly remembered, is the group of researches which he made in the field of what he himself called the "simpler natural bases".

At the time when he took up his post in the Wellcome Laboratories the active principle of the suprarenal gland, adrenaline, had recently been isolated and its constitution had been determined. The chemistry of this substance naturally attracted much attention and Barger, in collaboration with H. A. D. Jowett, took up the attempt to synthesize it. In this he was not completely successful, but his efforts were embodied in an interesting paper (Barger & Jowett, *J. chem. Soc.* 1905, **87**, 967) on the synthesis of compounds closely related to adrenaline. These experiments led first to some interesting chemical work on the action of thionyl chloride on methylenedioxybenzenes (*J. chem. Soc.* 1908, **93**, 563); the much more important outcome of the experiments was, however, the interest which they aroused in Barger's mind in the physiological action of relatively simple basic compounds. This phase of his work, throughout which he collaborated with Dale, began with a return to the study of ergot of which he had already investigated the alkaloid content; his isolation of ergotoxine had done something to explain the physiological activity of extracts of ergot, but still left much unaccounted for; Barger first showed (*J. chem. Soc.* 1909, **95**, 1123) that a part of the unidentified activity was due to the presence of tyramine, which compound he also synthesized; later (Barger & Dale, *J. chem. Soc.* 1910, **97**, 2592), he isolated the still more active substance histamine from the same source.

In 1911 (Barger & Dale, *J. Physiol.* 1911, **41**, 499) Barger demonstrated for the first time the occurrence of histamine in an animal tissue (gut); later developments in physiology and pathology have given this observation an importance which could not at the time be appreciated. In connexion with this it may be mentioned that in continuation of Barger's work his pupil Ewins was subsequently able to isolate acetylcholine from a particular ergot extract, and this compound also has since proved to have a profound physiological importance. The physiological implications of one aspect of this work, as they were then apparent, were treated in the classical paper of Barger & Dale (*J. Physiol.* 1911, **41**, 19) in which the general conception of sympathomimetic amines was developed.

During this period Barger published several other papers dealing with compounds allied to those under discussion; among these may be mentioned the synthesis of hordenine, the alkaloid of barley (*J. chem. Soc.* 1909, **95**, 2193) and of hypaphorine, the betaine of tryptophan (Van Romburgh & Barger, *J. chem. Soc.* 1911, **99**, 2068), which occurs in the seeds of *Erythrina hypaphorus*.

With Ewins (*J. chem. Soc.* 1911, **99**, 2336) he showed that ergothioneine, a sulphur-containing substance which had been isolated by Tanret from ergot, was the betaine of thiohistidine, whilst with Tutin (*Biochem. J.* 1918, **12**, 403) he was able to prove by synthesis that carnosine, which is a quantitatively important extractive of muscle, had the structure of  $\beta$ -alanylhistidine.

In later years Barger made another contribution in the field of amino-acid chemistry when he proved the constitution of the amino-acid methionine which had been discovered by Mueller in 1923. He synthesized this compound first in 1928 (Barger & Coyne, *Biochem. J.* 1928, **22**, 1417) and later by an improved method (Barger & Weichselbaum, *Biochem. J.* 1931, **25**, 997).

Of less immediate interest to biochemists, but of outstanding chemical importance, are the contributions which Barger made to the chemistry of

alkaloids; in his later years indeed work in this field occupied the greater part of his attention. His studies of alkaloids have been reviewed in detail in another place,<sup>1</sup> but brief mention may be made here of his more conspicuous achievements.

Barger's early study of the alkaloids of ergot, which was made in collaboration with F. H. Carr, has already been referred to. At the time when this study was begun considerable confusion existed concerning the physiologically active substances produced by ergot. The only crystalline alkaloid known to be extractable from the fungus was the ergotinine which had been discovered by Tanret, and this was physiologically inactive. Barger & Carr (*J. chem. Soc.* 1907, **91**, 307) described the isolation of a new alkaloid,  $C_{35}H_{41}O_6N_5$ , the salts of which could be obtained crystalline, and which showed a high degree of physiological activity. They showed, moreover, that the new alkaloid, which they named ergotoxine, was easily convertible into ergotinine and thus observed the first example of what has since turned out to be a general characteristic of all ergot alkaloids, namely that they occur in isomeric pairs, the members of which are interconvertible by simple means with large changes in specific rotation and physiological action.

Great physiological interest attaches to the work of Barger & Stedman (*J. chem. Soc.* 1923, **123**, 758; 1924, **125**, 1373; 1925, **127**, 247) on physostigmine (eserine): by skilful degradation experiments and close reasoning the structure of this alkaloid was completely elucidated. Not only was this work an important chemical achievement but it laid the foundation of the later well-known investigations of Stedman on the physiological action of simpler but analogous urethanes, among which at least one substance (the prostigmine prepared by Aeschlimann) has proved to be of great pharmacological and therapeutic value.

Two alkaloids which attracted Barger's early attention were carpine and yohimbine. In the first case he published a useful preliminary paper (*J. chem. Soc.* 1910, **97**, 466) and more than twenty years later resumed work on the problem in collaboration with Robinson; in two further papers (Barger, Girardet & Robinson, *Helv. Chim. Acta*, 1933, **16**, 90; Barger, Robinson & Work, *J. chem. Soc.* 1937, p. 711) the constitution of the alkaloid was cleared up. The work on yohimbine consisted of a preliminary paper (Barger & Field, *J. chem. Soc.* 1915, **107**, 1025) on the degradation of the alkaloid, followed much later (Barger & Scholz, *J. chem. Soc.* 1933, p. 614) by the highly significant recognition of harman among the degradation products which immediately fixed the arrangement of three out of the five rings composing the structure; finally (Barger & Scholz, *Helv. Chim. Acta*, 1933, **16**, 1343) identification of further degradation products permitted the entire skeleton of the alkaloid to be formulated with tolerable certainty.

During the years 1928–33 Barger made a series of researches on a group of aporphine alkaloids, and with a succession of pupils, among whom may be mentioned Silberschmidt, Girardet, Eisenbrand and Schlittler, he was able to explain completely the constitutions of laurotetanine, pukateine, laureline and lauropukine.

In quite recent years he had been engaged with J. J. Blackie on a systematic study of the *Senecio* alkaloids; at the time of his death one of his main interests lay in the work which he was doing on the difficult problem of the constitution of calycanthine.

Reference should be made to one other piece of work which Barger did, not because it led to any results of importance, but because at one time it exercised

<sup>1</sup> Obituary Notice, *J. chem. Soc.* 1939, p. 715.

a peculiar fascination over his mind. It has already been mentioned that when he was in Brussels he studied a glucoside saponarin; this substance had the unusual property of giving a blue compound with iodine similar to that given by starch. The observation attracted Barger's interest and he returned to the subject on several occasions in later years; he found that the property of forming such blue complexes with iodine was generally associated with the  $\gamma$ -pyrone group, and he published several papers dealing with the theory of the reaction and with constitutional factors affecting it.

Finally, mention must be made of two researches in which Barger played a less direct but nevertheless important part, namely, the work of the present writer on thyroxine and that of A. R. Todd on vitamin B<sub>1</sub> (aneurin). At the time when the writer started to work on thyroxine he had already left Barger's laboratory several years. This did not, however, prevent consultation by correspondence, and the keenness of Barger's interest and the freedom with which he gave of his experience did much to further the progress of the work. It is typical of Barger that, in spite of this, he so far belittled the value of his own contribution as only with difficulty to be persuaded to agree to joint publication of the final stage.

The recent distinguished work of A. R. Todd and his collaborators on the constitution and synthesis of aneurin was begun and largely carried through in Barger's laboratory in Edinburgh. In the early stages of this work particularly, his contribution was of great value.

Apart from his original papers in scientific journals Barger published several books; in his monograph on *The Simpler Natural Bases* which appeared in 1914 he collected a large amount of chemical and physiological information which was otherwise not easily accessible. In 1930 he published *Some Applications of Organic Chemistry to Biology and Medicine*, and in 1932 a text-book entitled *Organic Chemistry for Medical Students*: the book by which he will chiefly be remembered, however, is the monograph *Ergot and Ergotism*, which appeared in 1931. The writing of this masterly book was a labour of love extending over many years; in it every aspect of the subject is treated with a sureness of touch and a scholarly finish which make the work a model of its kind.

Barger's work was recognized by many distinctions in this and other countries. He was elected a Fellow of King's College, Cambridge, in 1904. In 1919 he was admitted to the Fellowship of the Royal Society; he served on the Council in 1930-2, and a few weeks before his death he was awarded the Davy Medal. At the time of his death he was a Vice-President of the Chemical Society, of which in 1936 he was Longstaff Medallist. In 1934 he received the Hanbury Medal from the Pharmaceutical Society.

In 1928 he held the Baker visiting Professorship of Chemistry at Cornell University, and during the same year he delivered the Dohme lectures at the Johns Hopkins University, Baltimore. The British Association elected him President of Section B (Chemistry) for their meeting in South Africa in 1929.

He received honorary degrees from the Universities of Liverpool, Padua, Heidelberg, Michigan and Utrecht, and he was an honorary or corresponding member of many foreign academies.

Barger's bilingual upbringing was the foundation of the exceptional linguistic capacity which he developed in later life and which reached its climax when he was able to address the International Physiological Congress in Moscow in 1935 in eight different tongues. This facility for acquiring foreign languages was combined with a great enthusiasm for travel, and enabled him to make many contacts with colleagues in other countries. These contacts, which developed in

several cases into close friendships, were of great value not only to himself but to the cause of mutual understanding between scientific workers which he had so much at heart. He was truly recognized as an international figure in Science, and the many honours which he received from foreign Universities and Academies indicate the high regard in which he was held by his colleagues abroad; this regard was shown in another way, however, which meant even more to him, namely, by the constant flow of foreign students who passed through his laboratory.

Barger's death will be felt not only as a loss to science but as a personal grief to his many friends. Those who were but slightly acquainted with him may not easily realize to what an extent he possessed the gift of true friendship. He was not the most patient of men and his manner at times made him seem far less patient than he really was, so that the first stages of acquaintanceship with him were the most difficult and there were even some who never succeeded in surmounting the initial barrier. Those who were admitted to his friendship, however, found it difficult to remember that any such barrier had ever existed; there can, indeed, have been few men who were willing to do so much in any way and at any time for their friends, or who were capable of showing so true a human sympathy.

To his pupils he gave without stint, both of his thought to their problems, and of his time in the laboratory to their practical difficulties. At the same time he did all in his power to encourage independence of thought and was at pains never to dissuade a student from testing a new idea experimentally even though he might himself feel sure from his own experience that the reaction would not "go".

It was indeed this love for experiment which was the keynote of Barger's attitude towards his work; he was instinctively mistrustful of hypotheses which were allowed far to outrun experimental support and this habit of mind coloured his whole outlook. It caused him to have strong leanings towards a mechanistic philosophy, departure from which, as he said in a discussion on the nature of life at the meeting of the British Association in 1929, he could not but regard as treachery to Science.

He himself possessed a high degree of experimental skill and had been at particular pains to perfect himself in the technique of working with small amounts of material long before such technique came to be generally regarded as a part of the equipment of an expert organic chemist. This capacity for the fine handling of small quantities was somewhat paradoxically combined with unsystematic, not to say untidy, habits in ordinary laboratory work.

Barger remained always a fundamentally simple-minded man; completely honest and straightforward in his outlook he never hesitated to express his views on any subject on which he felt strongly. He had little patience with formality and little respect for conventions although he would not willingly disregard conventions in such a manner as to offend others who might feel differently. It was inevitable that he should at times have irritated less outspoken people, and thus have made things more difficult for himself, but he was not easily deterred by the thought of such consequences if what he had to say were a matter of conviction. This essential sincerity made it impossible for him effectively to conceal his true feelings, even on those occasions when he judged that such concealment would facilitate the attainment of his object.

Fundamentally non-political in outlook, Barger was a man of liberal views with whom it was a ruling desire to break down all barriers such as those of nationality which interfere with the free intercourse of men of science; enough



has already been said to indicate how great were his services in the pursuit of this idea, and in these times particularly the measure of success which he achieved remains an encouragement.

No account of Barger would be complete without reference to the warm hospitality of himself and Mrs Barger, not only to friends and colleagues, but to students who were working with him; for many who have passed through his laboratory the remembrance of this will be one of their happiest recollections.

Barger has died as he would have wished while still in full activity; he leaves to his friends the memory of a beloved personality whose loss is a grievous blow, and to his pupils an inspiration which will endure throughout their lives.

I am indebted to the Chemical Society for permission to reproduce here much material from the more comprehensive Obituary Notice which has already been published in their *Journal*.

C. R. HARRINGTON.

### SERAFINO BELFANTI

1860-1939

BORN at Castelletto Ticino (Italia Settentrionale) on 28 March 1860, Belfanti took his degree in medicine at the University of Turin in 1886, remaining there as assistant in the Institute of Physiological Chemistry and later in the General Medical Clinic, then under the direction of Camillo Bozzolo. Here in 1889 he carried out his first investigations, in the then rapidly expanding field of Bacteriology, on the adaptability of the tetanus bacillus to aerobic conditions. He also studied the polymorphic character of this organism, the diffusion of the diphtheria bacillus throughout the body and its localization in certain organs (broncho-pneumonia) whilst he drew attention also to the existence of healthy diphtheria carriers. In 1894 he took his doctorate in Bacteriology. About this time the serum treatment of diphtheria was introduced and made such a stir as to decide the Medical Association of Lombardy to found in Milan an institute for the preparation of antidiphtheritic serum. Belfanti was called to be the first director and from then onwards devoted his whole life to this enterprise. Under his firm and paternal guidance the Serotherapy Institute of Milan grew from its humble beginnings in a few small rooms to the vast building of to-day, a veritable workshop concerned with the production of most of the country's sera, vaccines, chemotherapeutic and biological agents. Owing to the personal enthusiasm and passion of Belfanti for investigation the Institute became not only a huge productive centre but a home for workers imbued with a love of scientific research. Belfanti's own publications, numbering about 150, extend into bacteriological, immunological and biochemical fields, and are characterized by originality, restraint and conciseness in exposition. Always there stands out the attitude of the biochemist. As early as 1898 with his assistant Tito Carbone he commenced a careful biochemical study of the nature of antitoxins, and after numerous experiments on the fractionation of antitoxic sera reached the conclusion that antitoxins in horse serum are associated with globulins precipitated by half saturation with ammonium sulphate. Time has brought numerous confirmations of this discovery. Again in collaboration with Carbone, Belfanti showed for the first time that red corpuscles could act as antigens, an observation which clarified the subject of haemolysis.

Towards the end of 1926, when he had passed his sixtieth year, he gave fresh proof of his passion for research by initiating with his pupils and the chemist Contardi a notable series of experiments on cytolytic products of degradation of phosphatides produced by the action of special ferments (lecithinases), and on the differentiation and classification of phosphatidases. Some of the latter results were published in the *Biochemical Journal*, organ of the Biochemical Society, to which Belfanti was proud to belong.

His attainments received widespread recognition. He was vice-president of the Reale Istituto Lombardo di scienze e lettere, member of the executive of the Consiglio Nazionale delle Ricerche (National Research Council) and of many academies and scientific societies in Italy and abroad, including the Deutsche Akademie der Naturforscher, the Biochemical Society and the Indian Academy of Science. In 1934 he was nominated Senator of the Kingdom of Italy. In 1929 he founded the Italian section of the International Society of Microbiology with the object of assisting Italian bacteriology and aiding in its diffusion abroad.

The name of Serafino Belfanti will remain indissolubly bound to the growth of Bacteriology and Immunity. His faith in work, his cordiality and simplicity of character, his frank and friendly smile and lavish hospitality will long remain as precious memories to his pupils, friends and colleagues.

On 6 March 1939, after a brief illness, Belfanti's illustrious and fruitful career came to an end. Up to the last he interested himself in the work of his Institute, suggesting experiments and stimulating ideas until a few days before his death. He was buried in the family tomb at Castelletto Ticino, the land of his birth which he loved so well.

# CVI. THE GLYCOLYTIC ACTIVITY OF BRAIN

## I. MACERATED BRAIN TISSUE

BY ALEXANDER GEIGER AND JONATHAN MAGNES

## II. EFFECT OF CYTOLYSIS, THE PRESENCE OF A GLYCOLYTIC INHIBITOR

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## I. MACERATED BRAIN TISSUE

KNOWLEDGE of brain glycolysis compares unfavourably with that available for muscle. As yet, none of the intermediary steps of brain glycolysis is known with certainty and our knowledge of the coenzymes involved in brain glycolysis is very incomplete. In view of the conflicting statements in this field, we have here investigated the effects on brain glycolysis of general experimental conditions, of coenzymes and of certain of the known intermediary products of muscle glycolysis. It will be shown here that none of the substances which are intermediary products of muscle glycolysis can be regarded as such in glycolysis with intact brain cells. No alternate indication, however, of the intermediary steps involved in brain glycolysis can be given on the basis of these experiments.

Since finishing this work, cell-free extracts of brain with high glycolytic activity have been obtained. The results obtained with extracts—which differ somewhat from those obtained with intact cells—will be described in a later paper. Consequently, we wish to emphasize that when the present work was done, only intact brain cells were found to be glycolytically active; therefore the conclusions reached in this paper are valid for intact brain cells only.

*Methods.* The technique used was mainly that described previously [Geiger, 1935]. All the experiments were carried out on 2–6 months old albino rats, unless otherwise stated. Glucose was determined by Shaffer & Somogyi's method [1933]. Later the more recent modification [1937] of this method was used. Cozymase was prepared by Euler's method and later according to Meyerhof & Ohlmeyer [1937]. The adenosinetriphosphate<sup>1</sup> was prepared according to Barrenscheen & Filz [1932]. Warburg manometers were used for the measurement of respiration. Lactic acid was determined by the method of Friedemann & Graesser [1933].

*The effect of rat age and the season on brain glycolysis.* Macerated brain cortex of young rats shows a higher glycolytic rate than that of old rats. Table I contains a set of parallel experiments with macerated brain cortex from rats 2–3 months old and from rats more than a year old. In all the experiments, a measured amount of macerated brain was suspended in a known amount of

<sup>1</sup> Henceforth adenosinetriphosphate will be abbreviated AP, creatinephosphate CRP, reduced glutathione GSH, and oxidized glutathione GSSG.

*M*/15 phosphate buffer containing 200 mg. per 100 ml. of glucose, 50 mg. per 100 ml. of adenosine triphosphate and 50 mg. per 100 ml. of glutathione, all at pH 7.1.

Table I

Dilution of the tissue macerate	mg. lactic acid formed by 100 g. brain per hr.	
	Old rats	Young rats
1 : 100	340	510
2 : 100	340	530
3 : 100	338	480
4 : 100	330	435
5 : 100	320	410
6 : 100	300	375

The seasonal variation in glycolytic activity is also noteworthy. Higher values were obtained with our rats during the months of July and August. 120 determinations, under otherwise identical conditions, showed an average lactic acid production of 465 mg. per 100 g. tissue per hr. in winter and spring, as compared with 613 mg. during the late summer months.

*The effect of dilution of the tissue.* As Table I shows, dilution increases the relative glycolytic power of macerated brain cortex. In these experiments only the concentration of the tissue was varied.

We were also able to confirm Ashford's [1934] observation that tissue slices possess a much higher glycolytic power than macerated tissue.

*The effect of the composition of the medium.* It was observed by Ashford [1934] that the rate of brain glycolysis is lower in phosphate buffer than in bicarbonate Ringer. We are able to confirm this. We found, however, that if AP is added to macerated brain cortex in phosphate buffer, the glycolysis reaches almost the same level as it does in bicarbonate Ringer with added AP. This is illustrated in Table II.

Table II

Reaction-media: all at pH 7.2	mg. lactic acid formed by 100 g. tissue in 1 hr.
<i>M</i> /15 phosphate buffer	370
<i>M</i> /15 phosphate buffer and AP	550
Bicarbonate Ringer	560
Bicarbonate Ringer and AP	590

*Glycolysis with various brain cortex preparations.* In previous experiments [Geiger, 1935] extracts of brain cortex were made which had very little glycolytic activity in comparison with that of cortex macerate. Ashford [1934] was unable to obtain active extracts of brain cortex. Mazza & Malaguzzi-Valeri [1935] as well as Euler *et al.* [1936], however, described experiments with brain extracts showing much greater glycolytic activity.

In the present experiments we were unable to obtain extracts more active than those described previously. The NaCl or KCl extracts, even after addition of activators, had only about 1/10 of the activity of the brain cortex from which they were prepared. It should be noted, moreover, that only the first extract of macerated brain is at all active. Further washings on the centrifuge with isotonic KCl or NaCl did not yield active extracts and did not impair the glycolytic activity of the brain macerate. Washing with water destroyed the activity of the macerate.

In the experiments presented in Table III, ice-cooled brain cortex was carefully macerated in an ice-cold solution of NaCl or KCl. The suspension was

centrifuged and the residue washed several times more in the centrifuge. The first extract or the first and second extracts combined were made up with buffer solution to the same volume as the residue. In this way corresponding amounts of diluted extract and of the washed tissue suspension were obtained for the experiments.

Table III

	mg. lactic acid formed by 100 g. tissue per hr.	
Weight of tissue, g.	0.35	0.425
Unwashed + AP	444	—
Washed, no addition	—	242*
Washed + AP + GSH	382†	426*
Extract corresponding to same amount of tissue	—	6
First extract + AP + GSH	41	15
Tissue and extract + AP	421	—

\* Washed thrice.

† Washed once.

In later experiments it was observed that the destruction of the cell structure by distilled water, or by macerating with finely ground quartz-sand, completely destroyed the glycolytic activity of the brain. These experiments will be described in a later paper.

*Lactic acid formation from glycogen.* In Haarmann's [1932] and also in Ashford's experiments, the amount of lactic acid formed by macerated brain from glycogen was small as compared with that from glucose. Euler *et al.* [1936], however, obtained with brain extracts lactic acid formation from glycogen which sometimes equalled that obtained from glucose.

We repeated these experiments using both brain macerate and brain extracts, on the supposition that these conflicting results could be explained by the difference in the enzymic materials of these two preparations. Table IV shows the results.

Table IV

	mg. lactic acid formed by 100 g. brain cortex per hr.
Macerated brain suspension + AP + GSH + glucose	612
Macerated brain suspension + AP + GSH + glycogen	36
Macerated and washed twice + AP + GSH + glucose	589
Macerated and washed twice + AP + GSH + glycogen	31
Brain extract + AP + GSH + glucose	45
Brain extract + AP + GSH + glycogen	32

These experiments confirm the findings of Ashford [1933] according to which the amounts of lactic acid formed by macerated brain cortex from glycogen are small, as compared with the amounts formed from glucose.

In the extracts, the lactic acid formation from glycogen is as low as that in the macerated tissue, but since the glucose-splitting activity of the extracts is much lower than that of the macerated tissue, the relative rates of these two processes in the extract are different from those in the macerated tissue. In other words, the seemingly different behaviour of the extract and the macerate towards glycogen is merely due to the fact that under these conditions only a very small fraction of the glucose-splitting enzyme of the brain is active in the extract, while the activity of the glycogenolytic enzyme system is the same in the extract as in the original tissue.

Wilstätter & Rohdewald [1937] found that leucocytes and yeast transform glucose into glycogen prior to the formation of hexosediphosphate. In similar experiments made with brain no glycogen formation from glucose could be observed.

*The inability of macerated brain cortex to form lactic acid from added hexosediphosphate.* It was shown by Ashford [1933] and by Edlbacher *et al.* [1934] that brain cortex is able to produce small amounts of lactic acid from hexosediphosphate. Upon repeating these experiments with varying amounts of hexosediphosphate we found that the more hexosediphosphate added to chopped brain cortex, the greater the lactic acid formation. These experiments are contained in Table V.

Table V

	mg. lactic acid formed per hr. by 100 g. tissue	
	Exp. 1	Exp. 2
5 mg. hexosediphosphate at once	57	—
5 mg. hexosediphosphate after 1 hr.	62	—
10 mg. hexosediphosphate at once	100	121
10 mg. hexosediphosphate after 1 hr.	180	172
30 mg. hexosediphosphate at once	150	169
30 mg. hexosediphosphate after 1 hr.	380	376
60 mg. hexosediphosphate at once	—	250
60 mg. hexosediphosphate after 1 hr.	—	565

The amounts of hexosediphosphate indicated in this table were added to 10 ml. of brain suspension containing 0.4 g. of chopped brain cortex.

This singular behaviour of hexosediphosphate can be explained on the basis of the enzymic equilibrium between hexosediphosphate and dihydroxyacetone-phosphate, discovered by Meyerhof & Lohmann [1934]. According to these authors zymohexase is present in brain in much smaller quantities than in muscle. When hexosediphosphate is added to the brain macerate, triosephosphate is formed by the action of zymohexase in amounts depending—*ceteris paribus*—on the amounts of hexosediphosphate present. The rate of the process is proportional to the amount of enzyme present. The triosephosphate is in turn transformed into lactic acid artificially during the precipitation of the carbohydrates by copper-lime. This is illustrated in Table VI. Lactic acid and triosephosphate determinations were carried out immediately and at various time intervals after the addition of a known amount of hexosediphosphate to the brain pulp suspension. The triosephosphate was determined in the trichloroacetic filtrates according to Meyerhof & Lohmann [1934] by determining the amount of P liberated in 2N alkali at room temperature in 20 min.

Table VI

	mg. lactic acid formed	mg. triosephos- phate ( $P_3O_6$ )
With hexosediphosphate: At once	0.420	0.537
7 min. later	1.680	1.740
15 min. later	1.680	1.730
30 min. later	1.600	1.700
60 min. later	1.548	1.680

From these experiments it is evident that 7 min. after the addition of hexosediphosphate a certain amount of lactic acid is already "formed" and that this amount does not increase with time. On the other hand, estimation of triosephosphate shows it to be present in amounts corresponding to the amounts

of lactic acid found after treatment with copper-lime. It can therefore be safely deduced that chopped brain forms no lactic acid enzymically from hexose-phosphate and that the amount of lactic acid found in the experiments is derived from the artificial transformation of the triosephosphate which in turn is formed from hexosediphosphate by zymohexase. The relatively long time necessary for establishing the equilibrium between hexosediphosphate and triosephosphate in the brain pulp is due to the low zymohexase content of the brain. According to Meyerhof & Lohmann [1934] the zymohexase content of brain is only 1.6% of that in voluntary muscle, and in our experiments this small amount is diluted about 30 times. From the same experiments it may therefore be concluded that the brain is unable to form lactic acid from triosephosphate.

The inability of brain to convert hexosediphosphate into lactic acid led us to consider the possibility that lactic acid formation from glycogen by the brain is also effected via glucose. In this case the rate of lactic acid formation should depend on the diastatic activity of the brain. As the experiments in Table VII show, the addition of diastase to the brain pulp greatly increases the lactic acid formation from glycogen.

Table VII

	mg. lactic acid formed by 100 g. tissue per hr.	
	Exp. 1	Exp. 2
Brain pulp + glucose	557	472
Brain pulp + glucose + diastase	573	481
Brain pulp + glycogen	13	11
Brain pulp + glycogen + diastase	153	287

In these experiments 0.4 g. of brain pulp was suspended in 10 ml. of Ringer's solution. AP and glutathione were added to each flask.

*Autolysis of brain cortex.* In the hope of obtaining a suitable brain cortex preparation for studying the activators of brain glycolysis—brain extracts not being suitably active—we tried to eliminate the coenzymes, at least in part, by autolysing brain cortex in the absence of glucose. A series of experiments were made under different conditions, the results of which are summarized in Table VIII.

These experiments were made by macerating the brain in an isotonic NaCl solution, the required pH being obtained by the addition of HCl or NaHCO<sub>3</sub>. This suspension was shaken in a water bath at 37° for a known time in an atmosphere of N<sub>2</sub>, O<sub>2</sub> or air. Subsequently, equal amounts of the neutralized brain suspension were transferred in a measured amount of bicarbonate-Ringer to the experimental flasks containing glucose and the necessary additions. The flasks were then incubated at 37° for 1 hr.

Table VIII

	mg. lactic acid formed by 100 g. tissue per hr.			
	E. 105		E. 108	
	Glucose	Glucose + AP + GSH	Glucose	Glucose + AP + GSH
Before incubation	383	434	—	388
Incubated in N <sub>2</sub> for 1 hr. at neutrality	137	289	56	247
Incubated in O <sub>2</sub> for 1 hr. at neutrality	149	328	143	326

As Table VIII shows, autolysis in  $N_2$  inactivates more completely than in  $O_2$ , and brain macerate inactivated in  $O_2$  can be reactivated more completely by the addition of AP and GSH than when inactivated in  $N_2$ .

The rate of inactivation at different reactions was investigated in similar fashion. These experiments show that at an alkaline reaction the brain suspension becomes very rapidly inactivated and cannot be reactivated by the addition of AP and GSH. In an acid reaction the inactivation proceeds more quickly than at neutrality but reactivation can still be effected if the suspension is not autolysed for too long a time. In this connexion it is interesting to note that according to Giri & Datta [1936] brain phosphatases are inactive at neutrality and exhibit their greatest activity in the alkaline and acid ranges at about pH 5.0 and 9.0. In experiments during the late summer months we were unable to obtain as complete an inactivation by autolysis in  $O_2$  at neutrality, even when we extended the time of autolysis to 2 hr. At the same season the rate of glycolysis of the unautolysed tissue was found to be much higher than in the experiments made in the winter, spring and early summer months.

#### *Activators of brain glycolysis*

*Adenosinetriphosphate* was found to activate brain glycolysis. The experiments in Table IX show the rate of glycolysis with macerated brain cortex with and without the addition of AP.

Table IX

mg. lactic acid formed by 100 g. tissue per hr.

pH	Glucose only	Glucose + AP
6.00	140	145
6.40	325	342
7.00	441	533
7.50	389	580
8.00	279	555

At neutrality, the addition of about 3 mg. of AP to 10 ml. of brain suspension was sufficient for maximum activation. In the acid range, AP has practically no activating effect. Maximum *activation* occurs in the alkaline range. The maximum *rate* of glycolysis, however, is found at neutrality. In these experiments the fresh brain cortex of rats was used without previous autolysis, so that the brain itself contained some AP. The experiments were made in phosphate buffer, in which, as previously shown, the original amount of AP in the brain is not sufficient to cause full activation.

In order to be the better able to test the activating effect of several substances known to be present in brain macerate, we autolysed the fresh macerate at neutrality in air for 1 hr. at  $37^\circ$  as previously described. Table X contains

Table X

mg. lactic acid formed by 100 g. tissue per hr.

	Exp. 1	Exp. 7
Before autolysis + glucose + AP + GSH	482	486
Autolysed brain cortex + glucose	34	92
Autolysed + glucose + AP	223	410
Autolysed + glucose + CRP	40	100
Autolysed + glucose + hexosediphosphate	162	226
Autolysed + adenylic acid + CRP + glucose	—	95
Autolysed + glucose + AP + hexosediphosphate + CRP	289	421
Autolysed + cozymase + glucose	62	90



experiments of this type made with a suspension of macerated and autolysed brain cortex in bicarbonate Ringer.

To calculate the degree of activation in those experiments in which hexosediphosphate was also added, we subtracted the amount of lactic acid found in the flasks to which only hexosediphosphate was added, from the lactic acid content of the other flasks, which contained hexosediphosphate and other additions.

These experiments show that AP is able to bring about a maximal activation when added alone. None of the other substances caused the same degree of activation provided that the inactivation of the brain suspension was complete enough. The activating effect of hexosediphosphate seems to depend on the degree of inactivation of the tissue. The greater the inactivation of the brain suspension the less the activating effect of hexosediphosphate alone. After a complete autolysis in  $N_2$  the brain pulp could not be reactivated by AP. Cozymase had no activating effect on brain glycolysis.

Table XI

mg. lactic acid formed by 100 g. tissue per hr.

Additions to brain pulp	Fresh chopped brain in phosphate buffer		Autolysed brain	
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Glucose only	460	440	74	90
Glucose + AP	574	700	253	312
Glucose + hexosediphosphate	618	597	126	202
Glucose + AP + hexosediphosphate	618	—	260	307
Glucose + adenylic acid	465	427	68	95
Glucose + adenylic acid + hexosediphosphate	618	707	255	318

The experiments in the foregoing table show that adenylic acid alone has no activating effect but that the combination of adenylic acid and hexosediphosphate gives as high an activation as the addition of AP. Hexosediphosphate alone may sometimes also give full activation. This is probably due to the rapid dephosphorylation of the brain AP and the slower process of deamination of the adenylic acid.

#### *Transference of phosphorus to adenylic acid*

According to the findings of Parnas and co-workers, added hexosediphosphate is broken down in muscle to phosphopyruvic acid which transmits its phosphorus to the adenylic acid.

The following experiments show that in the brain this mechanism is different. Phosphopyruvic, added together with adenylic acid, is unable to activate glycolysis.

Table XII

mg. lactic acid formed by 100 g. brain per hr.

	Not autolysed	Autolysed brain	Autolysed brain
Glucose without addition	460	76	132
Glucose and adenylic acid	495	69	126
Glucose and adenylic acid and hexosediphosphate	618	397	402
Glucose and adenylic acid and phosphopyruvic acid	512	79	138
Glucose and adenylic acid and phosphoglyceric acid	—	83	140
Glucose + adenosinetriphosphate	—	382	398

Another proof that a brain suspension is unable to split phosphopyruvic acid is given by experiments in which the activating effects of small amounts of pyruvic acid and phosphopyruvic acid are compared. According to Mendel *et al.* [1931] small amounts of pyruvic acid (9–40 mg. per l.) added to brain cause an increased lactic acid production. The experiments in Table XIII show that the addition of phosphopyruvic acid is unable to bring about this activation with brain, although pyruvic acid does.

Table XIII

	mg. lactic acid formed from 100 g. brain per hr.		
	E. 145	E. 142	E. 163
Glucose and AP	495	—	427
Glucose and AP and pyruvic acid	615	825	759
Glucose and AP and phosphopyruvic acid	512	555	481

These experiments indicate that the transference of phosphate to adenylic acid, if such a mechanism exists in brain, is not via phosphopyruvic acid but by some other route. It has been shown by Neuberg [1935], by Lutwak-Mann & Mann [1935] and by Ohlmeyer [1935] that in yeast the direct transfer of P from hexosediphosphate to adenylic acid is possible. The experiments just described suggest the existence of this mechanism in brain.

### Phosphorylation

A further feature of brain glycolysis is the apparent absence of phosphoric ester formation. Ashford & Holmes [1929] and others were unable to demonstrate any phosphorylation. Euler *et al.* [1936], however, report experiments on brain extracts in which a disappearance of inorganic phosphate during glycolysis was observed.

In view of the strong activating effect of AP on brain glycolysis and of its role in muscle glycolysis as a phosphate carrier, it was felt necessary to reinvestigate this question. In our experiments made with and without the addition of AP and NaF, no phosphoric ester formation could be demonstrated. Moreover, in accordance with Ashford & Holmes' [1929] findings, a slight liberation of inorganic phosphate was found.

### The role of glutathione

In a previous paper [Geiger, 1935] experiments in which GSH had a marked activating effect on brain glycolysis were described. A similar effect had been observed by Quastel & Wheatley [1932] in yeast fermentation. Euler *et al.* [1936] confirmed the observation that GSH had a slight activating effect on lactic acid production by brain extract. These authors attribute the activating effect of GSH to its capacity for combining with heavy metals.

A large number of experiments carried out by us during the years 1935–6 have shown that the activating effect of GSH—although a constant factor in every experiment—is of a very different order of magnitude in different experiments. It may happen that in two experiments which are identical in every respect, except for the rats, the activating effect of GSH is as high as 100% in the one and not more than 10% in the other. Table XIV contains experiments selected at random from a large number of experiments made under different conditions.

Table XIV

	mg. lactic acid formed							
	Exp. A		Exp. B		Exp. C		Exp. D	
	No GSH	GSH added	No GSH	GSH added	No GSH	GSH added	No GSH	GSH added
Brain cortex of young rats in bicarbonate-Ringer	410	500	487	512	330	462	243	533
Brain cortex of old rats in bicarbonate-Ringer	320	426	229	346	274	306	222	451
Brain cortex in phosphate buffer pH 7.2	245	355	270	290	248	296	361	381
Brain cortex in phosphate buffer with added AP	385	492	309	411	367	416	239	500
Washed twice with 0.9% NaCl, then AP added	396	466	443	470	339	510	470	485

The only factor influencing the activating effect of GSH was the  $H^+$  concentration of the medium. The activating effect of GSH is largest as a rule at pH 6.4 and diminishes with increasing alkalinity. The behaviour of GSH towards pH is thus directly opposite to that of AP which activates more strongly on the alkaline side.

Nutrition is another factor which has some influence on the activating effect of GSH. The addition of GSH to the brain pulp of starved or vitamin  $B_1$ -deficient rats produces a more marked activation.

The possibility that the activating action of GSH was due to its binding the traces of Cu or Fe present in the brain pulp (or possibly in the reagents used) may be discarded on the basis of the relatively large amounts of Cu which are necessary to cause inhibition. In a series of experiments various amounts of Cu and Fe were added to the experimental flasks. Table XV shows that the addition of 0.04 mg.  $CuSO_4$  to 10 ml. brain suspension causes only a very slight inhibition of brain glycolysis. It is remarkable what large amounts of Cu are necessary to cause inhibition in brain macerate, as compared with muscle glycolysis. These experiments showing that small traces of heavy metals have no effect on brain glycolysis exclude the possibility that the activating effect of GSH on brain glycolysis is due to the binding of traces of Cu or Fe originally present in the macerate as suggested by Euler *et al.* [1936].

Table XV

	mg. lactic acid formed by 100 g. brain per hr.	
	$CuSO_4$	$FeCl_3$
No addition	565	581
With 0.40 mg. % heavy metal salt	520	—
With 1.00 mg. % heavy metal salt	460	567
With 2.00 mg. % heavy metal salt	470	—
With 20.00 mg. % heavy metal salt	410	—

#### *The effect of oxidized glutathione*

The statement was made by one of us [Geiger, 1935] that oxidized glutathione strongly inhibits muscle glycolysis and also inhibits brain glycolysis to a smaller extent. It appears from later experiments that this statement was erroneous. The error was caused by the presence of a relatively large amount of Cu in the glutathione preparation used. Later it was shown by Wagner-Jauregg & Rzeppa [1936] that minute amounts of Cu inhibit muscle glycolysis. It was also observed

later by us that the Cu liberated from our glutathione preparation on oxidation was sufficient to cause an almost complete inhibition of muscle glycolysis. When benzoin-oxime was added to precipitate the Cu liberated by oxidation, no inhibitory effect was observed. A recrystallized preparation of glutathione or a commercial one did not inhibit, or at most only very slightly when employed in large quantities. Table XVI shows the results of these experiments. (Benzoin-oxime, when added to the muscle preparation, had no effect on glycolysis.)

Table XVI

	mg. lactic acid formed by 100 ml. muscle extract in 1 hr.
50 mg. % GSSG containing Cu	181
50 mg. % GSSG containing Cu + benzoin-oxime	1120
50 mg. % recrystallized GSH oxidized	1210
50 mg. % commercial GSH oxidized	1220
Without GSSG	1180

## DISCUSSION

The experiments described in this paper show that the glycolytic process in the brain differs markedly from that in muscle. One difference is that cytolysis of brain cortex with distilled water causes complete inhibition of glycolysis. Another is the difference in the behaviour of the two systems towards inhibitors such as fluoride, iodoacetate or oxalate. All these substances inhibit both brain and muscle glycolyses. However, in the case of brain, when any of these substances is present, no phosphorylated intermediary products are formed, and glucose is not attacked at all. No phosphoric ester formation can be demonstrated in the course of brain glycolysis.

Copper ions, which according to Wagner-Jauregg & Rzeppa [1936] completely inhibit muscle glycolysis in concentrations of  $10^{-5} M$ , do not inhibit brain glycolysis in concentrations up to  $10^{-3} M$ . In this connexion Bauer's findings [1936], according to which  $Cu^{++}$  inhibits the action of zymohexase, may be of interest. Since, as has been shown in this work, brain cannot form lactic acid from hexosediphosphate or triosephosphate, the selective inhibition of the action of zymohexase would not inhibit brain glycolysis.

There is definite proof that the way of breakdown of glucose and the intermediary products involved depart in many respects from the scheme set up for muscle glycolysis. The present paper shows that no lactic acid can be formed in brain from added hexosediphosphate, phosphoglyceric acid or phosphopyruvic acid. Brain is unable even to hydrolyse phosphopyruvic acid. It was demonstrated by Johnson [1936] that  $\alpha$ -glycerophosphate and pyruvic acid cannot be regarded in brain as possible precursors of lactic acid.

It may be added that the absence of lactic acid formation in brain from added zymophosphates cannot yet be regarded as a conclusive proof against phosphorylation. As has been shown here, only intact cells are able to form lactic acid from glucose. Cytolysed brain suspensions, as will be shown in a later paper, are unable to attack glucose. It is therefore possible that the problems presented by living yeast—in which case phosphorylation is still under discussion—also apply to brain. Dickens [1936] has claimed that the entry of glucose into brain cells is accompanied by phosphorylation.

On the other hand, recent work by Needham & Lehmann [1937] and Needham & Nowinski [1937] has shown that hexosediphosphate does enter into embryonic cells, although practically no lactic acid is formed from it. The work of Needham

and his collaborators shows in many respects a striking similarity between embryonic and brain glycolyses.

It is however difficult to explain the activating effect of adenosinetriphosphate in a non-phosphorylating glycolysis. All our experiments show the absence of phosphorylation and the inability of brain to form lactic acid from phosphorylated compounds. On the other hand, adenosinetriphosphate which is considered as a transporter of P, activates the glycolysis in inactivated brain in some cases by as much as 300–400 %.

#### SUMMARY

1. The glycolytic rate of brain from adult rats is lower than from that of young animals.
2. The glycolytic rate in brain pulp increases with increased dilution of the tissue by isotonic saline solutions.
3. Higher glycolytic rates were obtained in Ringer's solution than in a phosphate buffer solution of corresponding *pH*. If adenosinetriphosphate is added to the phosphate solutions the glycolytic rate attains that reached in Ringer's solution.
4. Extraction with water destroys the glycolytic activity of brain. Repeated washing of brain pulp with isotonic solutions does not diminish its glycolytic power.
5. Only small amounts of lactic acid are formed by brain from glycogen. Brain extracts possess the same glycogenolytic activity as brain tissue. Owing to the low glycolytic activity of these extracts, the relationship—glycogenolysis: glucolysis is different in the extracts from what it is in the tissue.
6. No lactic acid is formed from hexosediphosphate by brain. The apparent lactic acid formation from hexosediphosphate is due to the slow formation of triosephosphate, which in turn is transformed into lactic acid during the analysis.
7. Brain pulp when incubated at 37° in N<sub>2</sub> or O<sub>2</sub> without addition of glucose becomes partially inactivated. The glycolytic power of the autolysed pulp may be partly restored by adenosinetriphosphate and glutathione. Adenosinetriphosphate may be replaced by a combination of adenylic acid and hexosediphosphate. Brain pulp inactivated at *pH* 8.0 cannot be reactivated by these substances.
8. Adenosinetriphosphate may be regarded as a coenzyme of brain glycolysis. Under the present experimental conditions, cozymase and creatinephosphate have proved to be ineffective.
9. It is indicated that P may be transferred by brain from hexosediphosphate to adenylic acid but not from phosphopyruvic acid.
10. Brain tissue is unable to dephosphorylate phosphopyruvic acid.
11. No phosphoric ester formation from inorganic phosphate can be demonstrated with intact brain cells.
12. The activating effect of glutathione on brain glycolysis is very irregular. It is however demonstrated that this activating effect is not connected with the presence of heavy metals.
13. A former statement, according to which oxidized glutathione inhibits glycolysis, was erroneous. The inhibition in these experiments was due to the presence of small amounts of Cu in the glutathione preparation.
14. It is suggested that the way of breakdown of glucose in brain departs in every respect from the way of breakdown of glycogen in muscle.

## II. EFFECT OF CYTOLYSIS, THE PRESENCE OF A GLYCOLYTIC INHIBITOR

In the first part of this paper it was reported that brain cortex loses all its glycolytic power if suspended in distilled water or if ground thoroughly with quartz sand. Case [1929] found that only intact brain cells are able to activate lactic acid formation from glucose by muscle extract. Dickens [1936] found that, following destruction of brain cells by grinding with sand, glucose oxidation ceases. Similar observations have been reported by Jowett & Quastel [1937].

It appears, however, that the necessity of intact cell structure for glucose breakdown is not limited to the brain cells. Irving [1927] stated that the integrity of kidney cells is essential for the utilization of glucose. The same view was also held by Case [1929]. The glycolytic activity of tumour cells is completely destroyed by freezing, grinding and by extracting with water according to Barr *et al.* [1928]. The same observation was made by the author on rat uterus (unpublished). According to Needham & Nowinski [1937] the destruction of cell structure abolishes glycolysis in the embryo.

Young [1929], Penrose & Quastel [1930] and Yudkin [1937] showed that cytolysis of certain bacteria results in the destruction of glucose dehydrogenase and of lactic dehydrogenase.

Brain extracts retain only a very small fraction of the glycolytic activity of the original tissue, the bulk of the activity remains in the tissue, and is not removed by further extractions [Part I]. Extraction with distilled water completely destroyed the glycolytic activity in the brain tissue.

Boyland *et al.* [1937] recently succeeded in activating a cell-free extract from "Crocker 180" tumour by adding relatively large amounts of cozymase and adenylic acid. These extracts produced lactic acid from glucose as rapidly as did the original tissue. These authors believe that tumour extracts during extraction are inactivated by nucleosidases liberated on destruction of the tumour cells. It appears therefore that the lack of glycolytic activity in tumour cell extracts is due to the destruction of coenzymes.

The earlier part of the present experiments was undertaken to ascertain the effects of cytolysis of brain tissue on the glucose breakdown and to study the nature of the inhibition. In the later part an inhibitor of glycolysis which is liberated from the brain on cytolysis is described.

### *The effect of suspension in water, of grinding with sand and of freezing, on the glycolytic power of brain cortex*

Brain cortex ground in a smooth-surfaced mortar and suspended in isotonic NaCl solution has only slightly lower glycolytic activity than slices prepared from the same brain. If, however, the brain is ground with the addition of fine quartz sand the activity of the resulting suspension decreases considerably, the decrease in activity depending on the thoroughness of grinding. A complete loss of activity results from suspending the brain pulp in distilled water or from repeated freezing and thawing of the brain pulp [Table I]. For these experiments rat brain hemispheres were hashed with scissors. Aliquot parts of the hash were treated in the manner indicated in Table I and were then suspended in four parts of water or in isotonic NaCl solution. After standing at room temperature for

10 min. the suspensions were distributed in Erlenmeyer flasks containing bicarbonate-Ringer, glucose, glutathione and AP. Lactic acid was determined chemically.

Table I

	mg. lactic acid formed by 100 g. brain in 1 hr.
Ground in smooth mortar with 0.9% NaCl	712
Ground with sand with 0.9% NaCl	384
Thoroughly ground with sand with 0.9% NaCl	95
Frozen and thawed twice with 0.9% NaCl	64
Ground in smooth mortar, suspended in distilled water	35
Ground in smooth mortar, suspended in 4% NaCl	375
Ground in smooth mortar, suspended in 12% sucrose	621
1 : 5 0.9% NaCl extract of brain	67
1 : 5 water extract of brain	4

In these experiments the amount of glucose which disappeared roughly corresponded to the amount of lactic acid formed. There was no phosphoric ester formation, but liberation of some inorganic phosphate was observed. It is to be noted, that, while the extracts prepared with istic solutions possessed some activity, no glycolysis at all was observed in the water extracts.

Dickens [1936] stated that after the destruction of brain cells by grinding with sand, Robison ester could still be oxidized, although glucose oxidation was no longer appreciable. Therefore in the following experiments (Table II) several substances were tried as glycolytic substrates in cytolysed brain. The experiments were made in the same way as those in Table I. One of these experiments is contained in Table II.

Table II

	mg. lactic acid by 100 g. in 1 hr.	ml. O <sub>2</sub> consumed by 100 g. in 1 hr.
Cytolysed brain with glucose	71	36
Intact brain pulp with glucose	680	720
Cytolysed brain with glycogen	43	—
Intact brain pulp with glycogen	39	—
Cytolysed brain with Robison ester	14	11
Intact brain with Robison ester	16	—
Cytolysed brain with hexosediphosphate*	0	0
Intact brain pulp with hexosediphosphate	0	0
Cytolysed brain with $\alpha$ -glycerophosphate and pyruvic acid	33	—
Cytolysed brain with lactate	—	46
Intact brain pulp with lactate	—	745
Cytolysed brain with succinate	—	900
Intact brain pulp with succinate	—	1095

\* The initial values were taken after 10 min. incubation at 37°.

The respiration of brain is greatly restricted on cytolysis. The O<sub>2</sub> uptake with glucose and with lactic acid is very small. No more O<sub>2</sub> was taken up with Robison ester than with glucose; the oxidation of succinic acid however was practically unaffected by cytolysis. The glyoxalase activity of brain was also unimpaired after cytolysis. The glycolytic activity of cytolysed brain could not be restored by the addition of adenosinetriphosphate and of cozymase. No appreciable decoloration of methylene blue could be observed with cytolysed brain in the presence of AP, or adenylic acid or cozymase. Boyland & Boyland [1935] stated that tumour extracts oxidatively deaminate adenosinetriphosphate and cozymase.

*The presence of a glycolysis inhibitor in cytolysed brain*

Cytolysed brain cells or an alkaline brain extract inhibit the glycolysis of muscle extracts and the fermentation of yeast extracts. This is the more remarkable since Case [1929] found that the addition of intact brain cells to muscle extracts enabled the latter to form lactic acid from glucose.

It was found by Winfield & Hopkins [1915] that pancreas contains a substance which inhibits lactic acid formation from glycogen in muscle. It was later demonstrated by Case & McCullagh [1928] that this substance is amylase. The pancreatic inhibitor did not inhibit lactic acid formation from hexosediphosphate, or from glucose when hexokinase was added to the muscle extract. The same observation was made by Barr *et al.* [1928]. As the following experiments show, the antiglycolytic substance in brain is not amylase although it is probably an enzyme.

*The inhibition by cytolysed brain of lactic acid formation in muscle extract from different substances*

Preliminary experiments have shown that small amounts of fresh brain pulp suspended in distilled water, when added to muscle extract, inhibit lactic acid formation from glycogen. Extracts of cytolysed brain, made with distilled water,

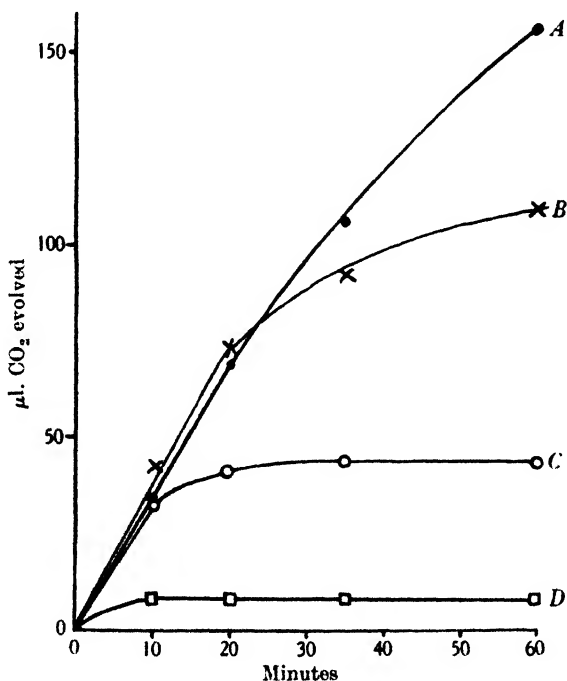


Fig. 1. The effect of cytolysed brain on glycolysis in muscle extract. A, no cytolysed brain; B, 0.2 ml. cytolysed brain susp.; C, 0.4 ml. cytolysed brain; D, 0.6 ml. cytolysed brain added to 2.5 ml. muscle extract.

showed very slight inhibitory action. Starting with small amounts of cytolysed brain it was observed that the inhibition of glycolysis was progressive. The rate of inhibition depended on the amount of cytolysed brain added, as shown in Fig. 1. In all experiments in which complete inhibition was observed the final



glycogen content of the reaction mixture was still high. This last fact indicates that the inhibiting factor is not amylase. The experiments are described in Tables III, IV and V. Brain was cytolysed by suspending the pulp in 5 vol. of distilled water. The extract from fresh muscles of rabbits or rats was prepared according to Meyerhof. In a large number of the experiments acetone-dried muscle extracts were used. The dry muscle powder was extracted with isotonic NaCl solution and was cleared by centrifuging. The glycolytic rate was determined chemically as well as by manometric methods. In several cases the glycogen content of the flasks was also determined at the end of the experiment. In all the experiments where lactic acid was determined chemically, 1 ml. of muscle extract was made up with *M*/15 phosphate buffer of *pH* 7.2, or with bicarbonate-Ringer, and with other additions to 5 ml. Glutathione and AP were added to each flask. For brevity's sake only one experiment from a larger number of uniform results is given.

Table III

	mg. lactic acid formed in 1 hr.	mg. glycogen found at start of exp.	$\mu$ l. CO <sub>2</sub> evolved in 1 hr.	mg. glycogen found at end of exp.
Muscle extract:	(1) Glycogen			
Without brain	2.74	156	122	38
With 0.04 g. cytolysed brain	1.21	138	46	29
With 0.06 g. cytolysed brain	0.18	129	0	24
	(2) Hexosediphosphate			
Without brain	1.89	—	108	—
With 0.04 g. brain cytolysate	1.50	—	91	—
With 0.08 g. brain cytolysate	1.04	—	35	—
With 0.12 g. brain cytolysate	0.29	—	0	—
	(3) $\alpha$ -Glycerophosphate			
With $\alpha$ -glycerophosphate	1.63	—	68	—
With 0.08 g. cytolysed brain	0.81	—	31	—
Cytolysed brain	0.44	—	11	—
	(4) $\alpha$ -Glycerophosphate and pyruvic acid			
Muscle extract	2.28	—	132	—
With 0.08 g. brain cytolysate	1.25	—	63	—
With 0.12 g. brain cytolysate	0.64	—	26	—

The amounts of cytolysed brain stated in the table were added to each ml. of muscle extract.

As Table III shows, the inhibitor cannot be amylase as there is still a considerable amount of glycogen left when inhibition is complete, and, as lactic acid formation is inhibited from both hexosediphosphate and from  $\alpha$ -glycerophosphate. Larger amounts of cytolysed brain are necessary to inhibit lactic acid formation from hexosediphosphate than from glycogen.

*Inhibition of lactic acid formation from glucose in muscle extract in the presence of hexokinase*

Cytolysed brain also inhibits lactic acid formation in muscle extracts from glucose in the presence of hexokinase. In these experiments acetone-dried muscle extract was used. The hexokinase was prepared according to Meyerhof [1927].

Table IV. *Inhibition of lactic acid formation from glucose in the presence of hexokinase*

	mg. lactic acid formed in 1 hr.
Muscle extract, glucose, AP and hexokinase	7.42
As above, with the addition of 0.1 g. cytolysed brain	0.66

*Inhibition of fermentation in yeast extract*

Fermentation in yeast extracts is also strongly inhibited by cytolysed brain. In one experiment, for instance, 289  $\mu\text{l. CO}_2$  were evolved without the addition of cytolysed brain, and only 20  $\mu\text{l.}$  in the presence of cytolysed brain.

*Effect of cytolysed brain on glycolysis of intact brain cells and on the fermentation in living yeast*

Glycolysis and fermentation of intact cells cannot be inhibited by cytolysed brain, hence the inhibitory substance cannot pass through cell membranes. An experiment of this type is described in Table V.

Table V

	$\mu\text{l. CO}_2$ evolved
Brain pulp in Ringer solution	123
As above, with 0.3 g. cytolysed brain	132
Living beer yeast	186
Living beer yeast with 0.3 g. cytolysed brain	194

*Effect of coenzymes*

By the addition of adenosinetriphosphate, of cozymase, of glutathione and of creatinephosphate it was attempted to reactivate muscle extracts which had been inactivated by cytolysed brain. Different amounts of the coenzymes were used. The maximum amount of cozymase added to 2.2 ml. fluid was 10 mg. of a pure product. In no case could any reactivation be observed. In these experiments, as in the former ones with brain cytolysate alone, an increased  $\text{CO}_2$  output was observed after adding cozymase. Chemical analysis showed, however, no corresponding increase in lactic acid formation. For the sake of brevity these experiments are not described here.

The facts described in this section indicate a difference in behaviour between tumour cells and brain tissue. Cozymase and adenylic acid do not reactivate cytolysed brain although they did reactivate tumour extracts in the experiments of Boyland *et al.* [1937]. On the other hand brain cells do not form lactic acid from hexosediphosphate, while according to Boyland & Boyland tumour extracts do, after the addition of adenylic acid and cozymase.

*Preparation of antiglycolytic extracts from cytolysed brain*

Cell-free aqueous extracts of cytolysed brain possess very slight antiglycolytic activity. Repeated washing of cytolysed brain with water only slightly diminishes its inhibitory power. Acid extraction has no inhibitory effect. On the other hand, alkaline extracts show a considerable inhibiting activity. Experiments were made as follows: finely ground pulp of fresh brain cortex was suspended in four parts of distilled water or in 0.1 *N* acetic acid or in 0.6%  $\text{NaHCO}_3$ . After 15 min. at room temperature, part of the suspension was pipetted off and the remaining part centrifuged. The clear supernatant extract was pipetted off and the residue filled up to its original volume. In a few cases this last procedure was repeated several times. To test the inhibitory effect of brain extracts, they were added to muscle extract containing glucose, hexokinase and AP. The experiment in Table VI was made manometrically. The total volume of fluid in the manometer vessels was 3.2 ml.

Table VI

	$\mu\text{l. CO}_2$ evolved in 1 hr.
Muscle extract, glucose, hexokinase, AP	118
With 0.2 ml. of cytolysed brain suspension (1 : 5)	0
With 0.5 ml. of water extract of cytolysed brain	109
With 1.5 ml. of water extract of cytolysed brain	85
With 1.5 ml. of acid extract of cytolysed brain	109
With 0.4 ml. of alkaline extract of cytolysed brain	35
With 0.6 ml. of alkaline extract of cytolysed brain	3

As Table VI shows, extracts possessing about a third of the inhibitory action of the original suspension may be obtained by extraction with 0.5%  $\text{NaHCO}_3$  solution.

*The effect of heating and of drying*

Heating of the cytolysed brain suspension or extract for 3 min. to  $100^\circ$  completely destroys its inhibiting power. On the other hand, in the heated suspension the presence of a substance which reactivates aged muscle extracts can be demonstrated. This activator can be best demonstrated on rat muscle extract which has been kept for 2 days previously in a refrigerator. One such experiment is shown in Table VII. It seems that this activator is not AP and not cozymase, as neither of these substances possesses the same activating effect as boiled brain extract. Precipitation and drying with acetone destroy a great part of the inhibiting effect of the extract. The inhibitory substance does not pass through collodion membranes.

Table VII. *Activating effect of heated brain extract*

	mg. lactic acid produced in 1 hr.
Aged rat muscle extract with AP and cozymase	39
Same as above with the addition of 0.4 ml. heated brain extract	81

*The effect of pH on inhibition*

The inhibitory effect of cytolysed brain on muscle glycolysis is greatest at pH 7.0–7.5. The experiment in Table VIII was made in *M*/15 phosphate buffer. The amount of cytolysed brain used was chosen so as to cause about 50–70% inhibition.

Table VIII. *Effect of pH on inhibition*

	mg. lactic acid formed in 1 hr.
pH 6.5 without inhibitor	1.42
with cytolysed brain	0.83
pH 7.0 without inhibitor	3.35
with cytolysed brain	1.31
pH 7.5 without inhibitor	4.70
with cytolysed brain	2.24
pH 8.0 without inhibitor	5.70
with cytolysed brain	4.14

DISCUSSION

The facts described in this paper prove that intact cell structure is necessary for brain glycolysis. By destruction of the cells a substance is liberated which inhibits glycolysis. No indication can be given of the nature of the inhibitory

substance although it is fairly well established that it is not amylase. It appears also that the inhibitor is different from the nucleosidase of tumour, described by Boyland & Boyland [1935]. While the nucleosidases reduce methylene blue in the presence of adenosinetriphosphate and of cozymase, no such reduction can be observed with cytolysed brain. The inhibitory action of cytolysed brain cannot be counteracted or the system reactivated by adenosinetriphosphate or by cozymase. On the other hand these substances reactivate tumour extract according to Boyland *et al.* [1937]. The glycolysis inhibitor of brain cannot be antiglyoxalase, since cytolysed brain possesses a considerable glyoxalase activity. Several facts, however, indicate the enzymic nature of the brain inhibitor. It is destroyed when heated for 2–4 min. to 100°. It does not pass through a collodion membrane. It loses part of its activity when dried and it has a pH optimum. The inhibition caused by cytolysed brain in muscle extract is a gradual one. The time at which the inhibition is complete depends on the amount of cytolysed brain present. This last fact indicates an enzymic destruction of a component of the glycolytic system.

As the inhibitory substance is indiffusible through collodion membranes it is evidently unable to penetrate cell membranes. In fact, cytolysed brain inhibits glucose fermentation in yeast extract, but it is unable to inhibit the fermentation of living yeast, and it does not inhibit glycolysis in a suspension of intact brain cells. These facts make it clear that extraction of the glycolytic system from brain causes at the same time liberation of an inhibitory substance which destroys the glycolytic activity.

#### SUMMARY

1. Brain tissue loses its glycolytic power if the cell structure is destroyed by grinding with sand, or freezing and thawing or by suspending it in water. The cytolysed brain cannot convert any of the zymophosphates into lactic acid.
2. The cytolysed brain tissue does not oxidize glucose. Lactate oxidation is also greatly reduced. Cytolysis has no effect upon succinate oxidation.
3. The glycolytic activity and the respiration of the brain which are lost by cytolysis cannot be restored by cozymase or by adenosinetriphosphate or glutathione.
4. The addition of small amounts of cytolysed brain to a glycolysing muscle extract inhibits the glycolysis of the latter. Lactic acid production, whether from glycogen, from hexosediphosphate, from  $\alpha$ -glycerophosphate and pyruvic acid, or from glucose with hexokinase, is inhibited by cytolysed brain.
5. Cytolysed brain inhibits the glucose fermentation of yeast extract.
6. Cytolysed brain does not inhibit the lactic acid formation by intact brain cells or the fermentation of living yeast.
7. Neither cozymase, adenosinetriphosphate, glutathione nor creatine-phosphate reactivates muscle extracts inhibited by cytolysed brain.
8. In boiled extract of brain the presence of a substance can be demonstrated which restores the glycolytic power of aged muscle extracts. The activator is neither cozymase nor adenosinetriphosphate.
9. With hypotonic  $\text{NaHCO}_3$  a cell-free extract of the glycolysis inhibitor can be prepared from cytolysed brain.
10. The inhibitor is destroyed by heat, and is partly destroyed by dehydration with acetone. It does not diffuse through collodion membranes.
11. The optimum pH for inhibition is 7.0–7.5.

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# CVII. METABOLISM OF SULPHUR

## VII. A QUANTITATIVE STUDY OF THE REPLACEABILITY OF *L*-CYSTINE BY VARIOUS SULPHUR-CONTAINING AMINO-ACIDS IN THE DIET OF THE ALBINO RAT<sup>1</sup>

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*(Received 11 March 1939)*

In experiments reported previously on the replaceability of *L*-cystine in the diet of albino rats with some partially oxidized derivatives [Bennett, 1937], the compounds fed were *L*-cystine disulphoxide, *L*-cysteinesulphinic acid and *S*-(guanyltio)-cystine.2HCl, which gives rise to *L*-cysteinesulphinic acid upon hydrolysis. *L*-Cystine disulphoxide proved capable of replacing *L*-cystine in the diet; *L*-cysteinesulphinic acid produced no growth; *L*-cysteinesulphinic acid resulted in a slight but definite increase in growth. These compounds were fed in sulphur equivalents that were in excess of the minimum amount necessary for maximum growth; therefore the results could not be interpreted quantitatively. In the present series of experiments, cysteine, cystine disulphoxide and methionine were fed, the basal diets being supplemented with smaller graded amounts of these amino-acids, in an attempt to demonstrate quantitative relationships in their ability to promote growth.

### *Preparation of compounds*

The compounds employed were: *L*-cystine (Merck); *L*-cystine disulphoxide (Merck); *L*-cysteine prepared from cysteine hydrochloride [Toennies & Bennett, 1936]; *dl*-methionine (Eastman), 98.0% purity according to sulphur determination;<sup>2</sup> *L*-methionine, isolated by Dr Toennies from egg albumin, 99.9% purity, Exp. 2; 97.0% purity, Exp. 4, according to sulphur determinations.<sup>2</sup> Arachin isolated from peanut meal<sup>3</sup> by the method of Johns & Jones [1916], 0.77% methionine and 0.92% cystine according to method of Kassel & Brand [1938].<sup>2</sup>

### EXPERIMENTAL

Albino rats, Wistar strain, 25 days old, were used as experimental animals. In Exps. 1-3 they were kept on Dyer & du Vigneaud's [1936] cystine-deficient basal diet containing 6% casein and 16% milk vitamin concentrate fed *ad libitum*. In Exp. 4 the basal diet was methionine-deficient; 15% arachin was used instead of casein as the basal protein and the dextrin was reduced to 24%; each rat received 100 mg. Harris vitamin B complex daily. Sufficient dry

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<sup>2</sup> I am indebted to Mr Thomas P. Callan for these determinations.

<sup>3</sup> I wish to thank the Planters Peanut Co., Norfolk, Va., for the gift of the peanut meal.

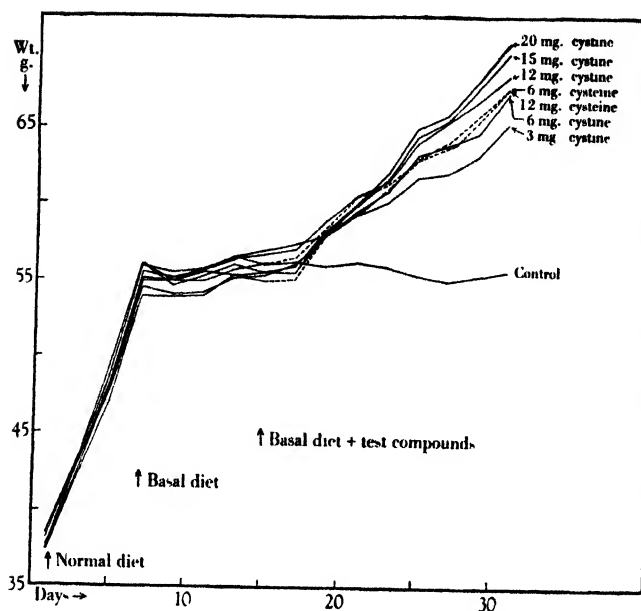


Fig. 1. Determination of the amounts of *L*-cystine and *L*-cysteine just adequate for maximum growth. Du Vigneaud's basal diet was used.

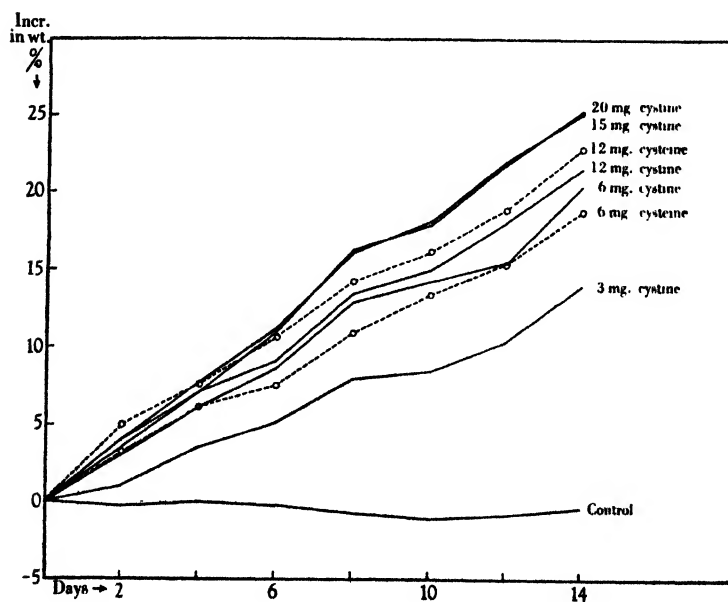


Fig. 1a. Fig. 1 recalculated, expressing the increase in weight in percentage of the weight at the beginning of the special diets.

mixture was made at the beginning of each experiment to last through the entire period. From this, fresh basal diets were prepared every 3 days. All food was kept in the refrigerator. The special compounds were fed individually in small pieces of butter, the control animals receiving the same amount of butter. On account of the extreme lability of the sulphur compounds, especially the cysteine, great care was taken to prevent decomposition. These butter mixtures were made once a week, keeping all instruments on ice during the mixing, and were kept in individual covered glass dishes in the refrigerator at 0°, the more labile at -10°. The animals were maintained on a normal diet for 8 days, at the end of which the basal diet was supplemented with the special compounds for a period of 2 weeks. The rats were weighed every other day and the average weight of the group plotted.

Exp. 1. *To determine the amount of cystine just adequate for maximum growth.* In the first experiment, 96 rats from 11 litters, 25 days old, were divided into 8 comparable groups of 12 animals each, 6 males and 6 females. After 8 days on the cystine-deficient diet, cystine was fed in addition to the basal diet in 20, 15, 12, 6 and 3 mg. portions daily to five groups respectively. Two additional groups received 12 and 6 mg. of *l*-cystine: one group was continued unsupplemented (Figs. 1, 1*a*). In Fig. 1*a*, the increase in weight is expressed in percentage of the weight at the beginning of the special diets. This allows the curves to start from a single point and brings out the true grouping which is slightly changed from that of the other graph. This experiment showed that 15 mg. of cystine added daily in excess of the basal diet produced maximum growth. It also brought out that the upper portion of the range was less suited to quantitative measurements than the lower in which slight differences in intake might produce greater spread of the curves. Therefore the scale of reference for the succeeding experiments was reduced to 12, 6, 3 and 1.5 mg. of cystine. The maximum amount of cystine added to these figures, due to the daily consumption of 5-10 g. of du Vigneaud's basal diet, would not exceed 1.8 mg. of cystine. These amounts of cystine are below the level of those used in most growth experiments reported in the literature. For instance, du Vigneaud, in his studies, fed the animals 20 mg. of cystine, or its sulphur equivalent, daily in addition to the cystine in the basal diet [Loring *et al.* 1933; Dyer & du Vigneaud, 1935; 1936]. A computation of the amounts of cystine ingested daily by the rats used by Womack *et al.* [1937] in their study of the growth-promoting potencies of cystine and methionine, show (their Tables II and III) that on an average approximately 12-16 mg. of cystine were consumed daily by each rat.

Exp. 2. *To determine the quantitative metabolic relationships of l-cystine, l-cystine disulphoxide, and l-methionine, as expressed by growth curves.* In the second experiment, 88 rats from 12 litters, 25 days old, were divided into 11 comparable groups of 8 animals each, 4 males and 4 females. After 8 days on the cystine-deficient diet, the various groups, with the exception of the control group, were supplemented daily. Four groups received 12, 6, 3 and 1.5 mg. of cystine respectively. The other six groups were fed daily 3.0 and 12.1 mg. of *l*-cystine; 3.7 and 14.9 mg. of *l*-methionine; and 3.4 and 13.6 mg. of cystine disulphoxide, respectively; amounts containing the sulphur equivalents of 3 and 12 mg. of cystine. Fig. 2 gives the customary type of growth curve; Fig. 2*a* shows the increase in weight expressed in percentage of the weight at the beginning of the special diets. As shown, all the curves representing 12 mg. equivalents (letter *D*) fall in a group as do those corresponding to 3 mg. (letter *B*). In both groups disulphoxide curves fall lower as the experiment progresses.



Exp. 3. *Repetition of Exp. 2 with dl-methionine in place of the l-form.* In the third experiment, 110 rats from 13 litters, 25 days old, were divided into 11

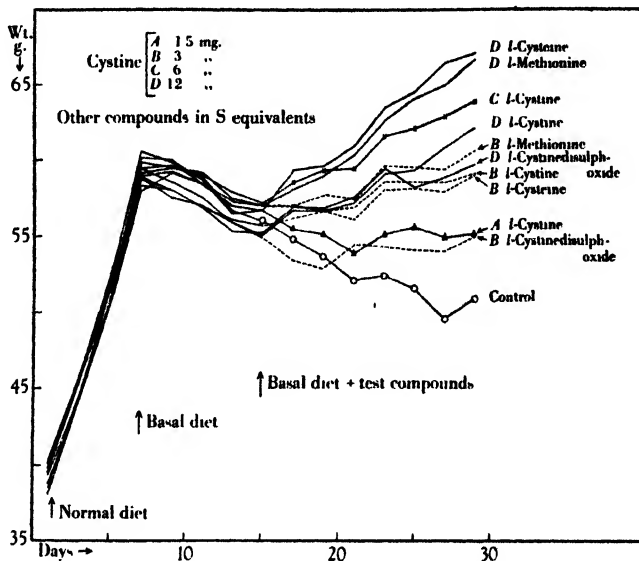


Fig. 2. Growth curves expressing the quantitative metabolic relationships of *l*-cystine, *l*-cysteine *l*-cysteine disulphoxide and *l*-methionine. Du Vigneaud's basal diet was used.

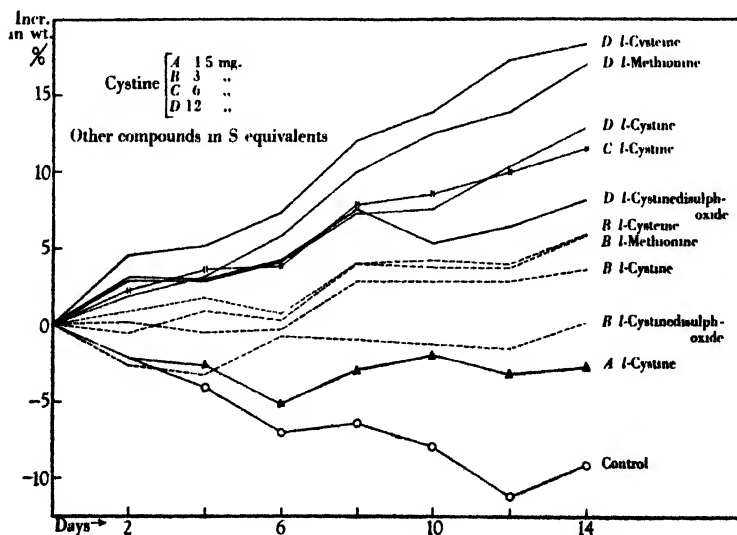


Fig. 2a. Fig. 2 recalculated, expressing the increase in weight in percentage of the weight at the beginning of the special diets.

comparable groups of 10 animals each, 5 males and 5 females. The various groups were treated as in Exp. 2; the compounds and amounts fed were the same, with the exception of methionine. The results are depicted in Fig. 3. The curves

representing the 12 mg. equivalents fall in a group as before and about the same growth is attained in all cases, indicating that with this intake all the compounds are present in sufficiently large amounts to insure maximum growth. In the group of curves representing the 3 mg. intake, however, a spreading occurs. The cystine disulphoxide curve, as before, is the lowest, dropping below that for 1.5 mg. cystine, nearer the probable location of a 1 mg. cystine curve. The 3 mg. *dl*-methionine curve falls slightly above the 1.5 mg. cystine curve.

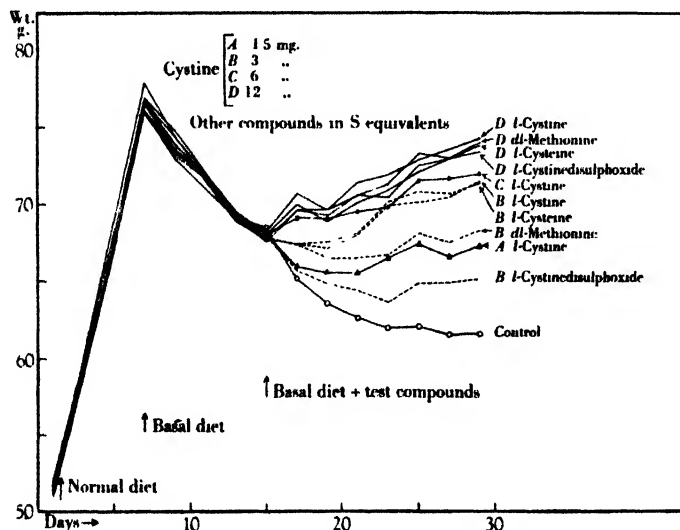


Fig. 3 Growth curves expressing the quantitative metabolic relationships of *l*-cystine, *l*-cysteine, *l*-cystine disulphoxide and *dl*-methionine. Du Vigneaud's basal diet was used.

Exp. 4. To determine whether *dl*-methionine is as readily utilized for growth as the *l*-form. A comparison of the growth curves of rats receiving the smaller amount of *l*-methionine in Exp. 2, and *dl*-methionine in Exp. 3, suggests that the *dl*-methionine may not be exactly the equivalent of the *l*-form. Since these curves are from separate experiments, it was decided to test this more adequately by feeding the *l*- and *dl*-forms of methionine in one experiment (4). The casein used in the basal diet provided 1.9 mg. of methionine for each g. of basal food. In an attempt to reduce this basal intake, arachin was substituted for casein since it is said to contain 0.54 % methionine [Baernstein, 1932] amounting to about 0.8 mg. of methionine per g. of basal food. The arachin isolated for the present experiment, however, showed 0.77 % methionine by Kassel & Brand's [1938] modification of Baernstein's [1932] method or 1.2 mg. of methionine per g. of basal food. Twenty-five male rats, 23 days old, from 5 litters were divided into 5 groups, one rat from each litter in each group. After 8 days on the methionine-deficient diet, the various groups, with the exception of the control, were supplemented daily. Groups B and C received 6 and 12 mg. of *l*-methionine, respectively, and D and E received the same amounts of *dl*-methionine daily. The approximate amount of the basal diet consumed per rat per day was determined by weighing daily the basal food given each group and the residual food, and dividing by the number of animals in the group (Table 1). This experiment was conducted on a methionine-deficient diet, with arachin as the basal protein. Fig. 4 gives the curves obtained.

Table I. *Daily basal food consumption per rat in g. Averaged over 2-day period. Exp. 4*

Days	Unsupplemented				
	Group A	Group B	Group C	Group D	Group E
1-2	4.1	4.5	5.2	4.7	4.6
2-4	4.9	5.4	5.7	5.4	5.4
4-6	4.7	5.2	4.9	5.5	5.0
6-8	4.4	5.9	5.9	5.3	5.2

	Daily supplement				
	Unsupplemented	l-Methionine, mg.		dl-Methionine, mg.	
	Control	6	12	6	12
8-10	4.5	6.4	6.2	5.4	5.8
10-12	4.7	6.8	5.4	5.2	6.2
12-14	5.0	6.2	6.3	5.5	6.6
14-16	4.8	5.8	5.7	6.0	6.6
16-18	4.7	6.2	6.2	6.1	7.3
18-20	4.4	6.3	6.3	5.9	7.4
20-22	4.8	6.6	6.8	6.4	7.8

Table II. *Relation of growth to food consumption. Averaged per rat over a 14-day period of feeding. Exp. 4*

Group	Daily supplement	Increase in weight g.	Food consumption g.	Gain per g. of food consumed g.
B	6 mg. l-methionine	11.2	88.6	0.126
C	12 mg. l-methionine	13.1	85.8	0.152
D	6 mg. dl-methionine	8.1	81.0	0.100
E	12 mg. dl-methionine	12.4	95.4	0.129

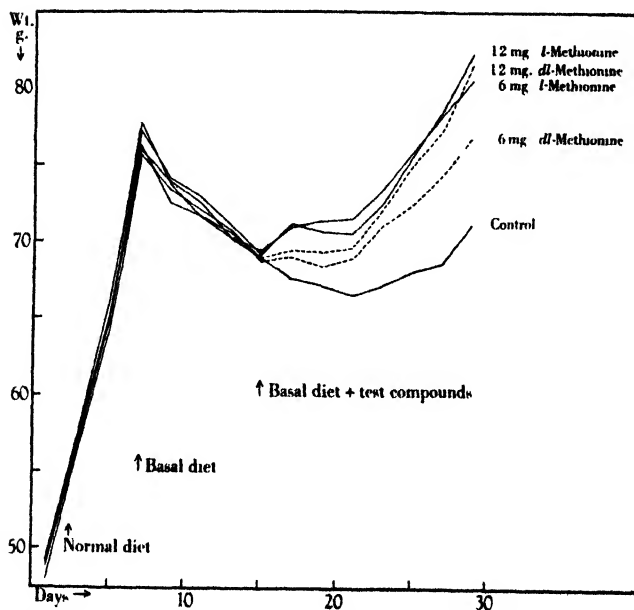


Fig. 4. A comparison of the growth-promoting properties of l-methionine and dl-methionine. The casein of du Vigneaud's basal diet was replaced by arachin.

## DISCUSSION

There has been some question as to whether the cystine-cystine relationship in metabolism is solely an oxidation-reduction relationship or whether hydrolytic cleavage of cystine also plays a part. In the former case, one molecule of cystine should be the equivalent of two molecules of cysteine; in the latter case, a one to two relationship would not hold. Shinohara & Kilpatrick [1934], working in acid solution without addition of heavy metals, reported that through hydrolysis and subsequent dismutation three molecules of cystine gave rise to five of cysteine and one of cysteic acid. This was in agreement with earlier work of Vickery & Leavenworth [1930] who worked with heavy metals in solution, whereby cysteine was removed from the mixture as rapidly as it was formed. Lavine [1937], also with heavy metals present, found that two molecules of cystine gave rise to three molecules of cysteine and one of sulphinic acid. In Exps. 2 and 3 the clear-cut separation of the curves into two groups representing those rats which received the equivalents of 12 mg. cystine and those which received the equivalents of 3 mg. indicates (Figs. 2, 2a, 3) that, under these conditions, two molecules of cysteine are fully the equivalent of one of cystine.

Whether oxidation of disulphoxide sulphur ever occurs *in vivo* is uncertain. Evidence from the present experiments shows that the formation of cystine from its disulphoxide can occur, although one molecule of cystine disulphoxide did not give rise to the same amount of growth as did one molecule of cystine. Possibly a series of reactions takes place in the body similar to that found in test-tube experiments by Lavine [1936], whereby three molecules of cystine disulphoxide, through hydrolysis and dismutation, give rise to four molecules of cysteinesulphinic acid and one molecule of cystine.

The results of Exp. 4 substantiate those of Exps. 2 and 3; the *dl*-methionine when fed in submaximal amounts did not produce quite as much growth as the *l*-form. Since both the *l*- and the *dl*-forms are oxidized to sulphate [Stekol, 1935], it might seem reasonable to suppose that they have equal growth-promoting properties. However, it is known that the readiness with which a compound is oxidized and its availability for growth do not run parallel in some compounds. For instance, du Vigneaud *et al.* [1934] found that *S*-methylcysteine is readily oxidized, but that feeding this compound does not bring about growth of rats on a cystine-deficient diet.

Considering the relation of growth to food consumption (Exp. 4, Table II) the gain in weight for each g. of food consumed is practically the same with an intake of 6 mg. *l*-methionine (group B) and 12 mg. *dl*-methionine (group E), 0.126 and 0.129 g. respectively. The lowest relative gain was attained with the 6 mg. *dl*-methionine supplement, or 0.10 g. for each g. of food ingested. An intake of 12 mg. *l*-methionine definitely increased the utilization of food for growth; these animals show 0.15 g. increase in weight for each g. of food consumed. Kik [1938] similarly reported a better utilization of food when a casein ration was supplemented by methionine than when supplemented by cystine. Animals on the 12 mg. *dl*-methionine, after several days, grew at approximately the same rate as those on 12 mg. *l*-methionine; it is attained, however, only by increasing intake, 85.8 g. of food for those on 12 mg. *l*-methionine compared with 95.4 g. for those receiving 12 mg. *dl*-methionine. Growth curves reported by Jackson & Block [1938] demonstrate this same tendency. Their rats receiving the *d*-methionine, increase their daily food consumption to a greater extent than those receiving the *l*-methionine, while gaining approximately the same number

of g. in weight. This tendency can be seen by a rough estimation of g. gained and daily food consumption given in Fig. 1 of their paper.

From Exp. 4 some idea of the absolute amounts of a mixture of cystine and methionine which will produce normal growth in albino rats may be obtained. Calculating the amounts of methionine and cystine consumed in the basal diet in addition to those fed as supplement, Group C had a daily intake of 19.3 mg. *l*-methionine and 8.5 mg. *l*-cystine; group E, 12 mg. *dl*-methionine, 8.2 mg. *l*-methionine, and 9.5 mg. *l*-cystine; group B, 13.6 mg. *l*-methionine and 8.8 mg. *l*-cystine. These three groups undergo practically the same increase in weight during the experimental period. The last group D, which received a daily intake of 6 mg. *dl*-methionine, 7.0 mg. *l*-methionine and 8.1 mg. *l*-cystine, grew somewhat less; therefore, in this case, we conclude that methionine and cystine were present in suboptimal amounts.

#### SUMMARY

Under the conditions of the experiments reported, the following quantitative metabolic relationships are indicated as expressed by growth curves:

One molecule of *l*-cystine is available from three molecules of *l*-cystine disulphoxide.

One molecule of *l*-cystine is equivalent to two molecules of *l*-cysteine.

One molecule of *l*-cystine is equivalent to two molecules of *l*-methionine.

*dl*-Methionine is somewhat less readily utilized for growth than is the *l*-form.

*l*-Methionine definitely increases the utilization of food for growth.

The absolute amounts of methionine and cystine, which permit normal growth in young albino rats on an otherwise adequate diet under the conditions of the last experiment, are estimated.

The author wishes to thank Dr Medes for her advice and criticism.

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# CVIII. STUDIES ON PROTEINASES OF SOME ANAEROBIC AND AEROBIC MICRO-ORGANISMS

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*CLOSTRIDIUM HISTOLYTICUM* secretes a proteinase, with a pH optimum of 7, which can be activated markedly by —SH compounds; and this activation can be increased by the addition of traces of heavy metal [Weil & Kocholaty, 1937; Kocholaty, Weil & Smith, 1938]. The results of the present study, an investigation of the proteinases of several other Clostridia, indicate that the proteinases of some other members of this genus have similar pH optima and activation characteristics.

Cell-free filtrates of *Pseudomonas fluorescens*, *Serratia marcescens*, *Bacillus mycoides* and *Staphylococcus citreus* contain proteinases which, although differing in their partial activation behaviour (with cysteine alone and  $\text{Fe}^{++}$  alone), as a whole resemble the proteinases of the members of the *Clostridium* group.

## EXPERIMENTAL

The bacterial proteinase solutions were prepared in the same manner as those previously reported [Weil & Kocholaty, 1937] by inoculating the bacteria into culture media and filtering through a Seitz filter after 18–24 hr. anaerobic incubation at 37°. For *Cl. histolyticum* and *Cl. Welchii*, horse meat broth was used; for *Cl. sporogenes*, a 3 % solution of Difco neopeptone; for *Cl. putrificum* and *Cl. botulinum*, beef heart infusion broth. The pH of the culture media was adjusted to about 7.4. The organisms used were obtained from the American Type Culture Collection. The strain of *Cl. histolyticum* was in the rough phase [Hoogerheide, 1937].

One ml. of *Cl. histolyticum*, 2 ml. of *Cl. Welchii* and 3 ml. of *Cl. sporogenes* or *Cl. botulinum* filtrates, respectively, were taken for each determination. To this amount of bacteria-free filtrate, either 10 mg. of cysteine-HCl (neutralized), 0.6 ml. of 0.1 *N*  $\text{FeSO}_4$ , or both, were added. After the addition of 3 ml. of citrate-phosphate buffer at the proper pH, 3 ml. of a 3 % gelatin solution of the same pH were pipetted into the reaction mixture. The total volume was made up to 10 ml. After an incubation of 22 hr. in a hydrogen atmosphere at 37°, the increase of amino groups was measured by the Van Slyke method, and the results were expressed in ml. of 0.05 *N* KOH. All the filtrates of the Clostridia were practically free of peptidases, and the activities measured in this way were attributable to the proteinase action alone.

From Table I it can be seen that the proteinases of the Clostridia resemble very much, with respect to pH optima and activations, the proteinase of *Cl. histolyticum*, already described [Weil & Kocholaty, 1937].

Maschmann [1938, 1] stated that the cell-free filtrate of *Cl. Welchii* without activation could hydrolyse only gelatin, although, when activated by —SH compounds, clupein was hydrolysed. The other protein substrates that he

Table I. *Activation and pH optima of the proteinases of some Clostridia measured on gelatin*

		pH...	4	5	6	7	8
Activator		Activity in ml. of 0.05N KOH					
<i>Cl. histolyticum</i>	None	0.0	0.56	3.52	4.32	3.62	
	Cysteine	0.0	1.20	4.84	5.62	4.98	
	Fe <sup>++</sup>	0.0	0.52	3.20	4.00	3.08	
	Cysteine-Fe <sup>++</sup>	0.10	1.74	5.82	7.16	5.64	
<i>C. Welchii</i>	None	0.04	—	0.94	1.20	1.00	
	Cysteine	0.20	—	1.96	2.24	2.04	
	Fe <sup>++</sup>	0.24	—	3.22	3.70	3.12	
	Cysteine-Fe <sup>++</sup>	0.24	—	3.00	3.28	2.74	
<i>Cl. sporogenes</i>	None	0.11	—	2.10	2.44	2.01	
	Cysteine	0.14	—	3.06	3.58	3.12	
	Fe <sup>++</sup>	0.22	—	4.01	4.22	3.82	
	Cysteine-Fe <sup>++</sup>	0.30	—	5.81	6.46	5.76	
<i>Cl. putrificum</i>	None	0.00	—	1.37	1.65	1.01	
	Cysteine	0.09	—	1.72	2.04	1.29	
	Fe <sup>++</sup>	0.32	—	2.41	2.81	1.68	
	Cysteine-Fe <sup>++</sup>	0.22	—	2.16	2.36	1.64	
<i>Cl. botulinum</i>	None	0.08	—	0.49	0.77	0.56	
	Cysteine	0.17	—	0.67	0.97	0.76	
	Fe <sup>++</sup>	0.30	—	0.83	1.18	0.98	
	Cysteine-Fe <sup>++</sup>	0.24	—	0.81	1.16	0.99	

investigated (ovalbumin, casein, Witte peptone) were but slightly hydrolysed. The proteinase responsible for gelatin hydrolysis was found to be secreted into the medium in the early stages of bacterial growth. However, the proteinase which induces the hydrolysis of clupein was described as appearing only late in incubation, presumably after autolysis of the bacteria had occurred. From this, Maschmann assumed that this latter proteinase was an intracellular one. Similar conclusions were reached [Maschmann, 1938, 2] in connexion with the proteinases of *Cl. histolyticum* and *Cl. botulinum*.

Because of this description of an intracellular proteinase which promotes the hydrolysis of clupein, and of our previous negative results in regard to this point [Weil & Kocholaty, 1937; Kocholaty, Weil & Smith, 1938], careful attention has been given, in the present study, to factors affecting autolysis.

A cell-free filtrate of *Cl. Welchii*, obtained from a 16 hr. culture, was used as a source of proteinase. At this time, 16 hr. after inoculation, practically all of the bacteria were living and dividing, and consequently, any enzyme present in the culture filtrate may be presumed to have been produced extracellularly. Two ml. of the *Cl. Welchii* filtrate were used for each enzyme activity determination. The activations were carried out as described above. After the addition of 3 ml. of citrate-phosphate buffer at pH 7.0, 3 ml. of a 5 % solution of gelatin, clupein sulphate, Witte peptone or casein at pH 7.0 were added. After 20 hr. of anaerobic incubation at 37°, the increase in amino groups was measured by the Van Slyke method. In the case of casein, alcoholic titration was used and the buffer was omitted, because of its disturbing effect on the titration. The pH, however, was adjusted in this special case.

As Table II shows, the proteinase of *Cl. Welchii* readily hydrolysed all the investigated proteins, and in every case a marked activation was obtained with cysteine-Fe<sup>++</sup>. The similar behaviour of the enzyme-activator system toward the investigated protein substrates did not indicate a number of proteinase actions. This enzymic picture resembles very much that obtained in our previous work [Weil & Kocholaty, 1937] on the proteinase of *Cl. histolyticum*.

Table II. *The decomposition of various protein substrates by Cl. Welchii proteinase*

Substrate	Activator			
	None	Cysteine	Fe <sup>++</sup>	Cysteine-Fe <sup>++</sup>
	Activity in ml. of 0.05N KOH			
Gelatin	0.84	1.91	1.01	1.91
Clupein	0.57	1.65	0.77	2.19
Witte peptone	0.60	0.00	0.10	1.40
Casein	0.56	1.28	0.76	1.32

A recent paper by Berger *et al.* [1938] also indicates a possible importance of reducing substances for the peptidase system of anaerobic bacteria. A similar observation was reported by Maschmann [1938, 3], and he made the suggestion that the activation observed by us on the proteinase of *Cl. histolyticum* was due to the peptidase system. However, such an explanation does not hold for the proteinases of Clostridia. Bacteria-free filtrates of the *Clostridium* group after 1 day's cultivation were found to contain practically no peptidase, whether tested for in the presence or absence of the activator system. The peptidases, as has been shown [Kocholaty, Smith & Weil, 1938], are intracellular, and appear in the bacterial filtrate only after the autolysis of the bacteria.

Further evidence that the activation is due to the proteinase action was given by the liquefaction of gelatin by the proteinase of *Cl. histolyticum*. The

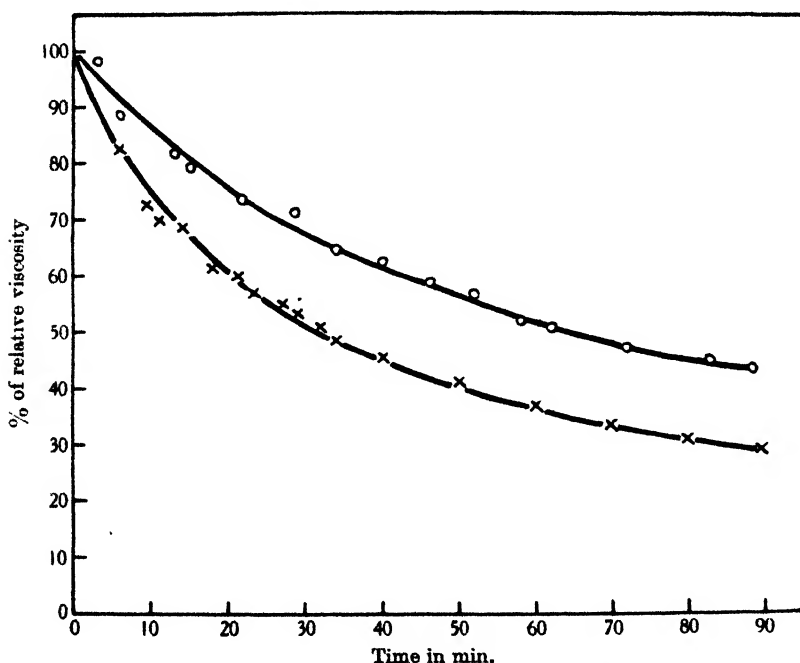


Fig. 1. ○—○ Without activator. ×—× With activator.

hydrolysis of gelatin by *Cl. histolyticum* proteinase, measured by the viscosimetric method, was markedly activated by cysteine-Fe<sup>++</sup>. This method of proteinase determination measures the very first step of protein breakdown, one



which is indeterminable by any chemical method. The activation of this first step of hydrolysis cannot, therefore, be explained by a peptidase action. One ml. of *Cl. histolyticum* filtrate, diluted 1:80, and 3 ml. of 3 % gelatin solution at pH 7.0 were used, the total volume being 4 ml. The activation was carried out as usual. The drop in the viscosity was measured at 37° and is expressed in percentage of the original viscosity (Fig. 1).

*Characteristics of the proteinases of some aerobic bacteria*

For the preparation of proteinase of *B. mycoides*, a pure culture was inoculated into a medium containing 0.3 % beef extract, and 0.5 % yeast extract. After an aerobic incubation of 5 days at 37°, the culture was filtered. Bacteria-free proteinase solutions of *Staph. citreus*, *Ps. fluorescens*, *S. marcescens* and *Proteus vulgaris* were obtained through inoculation of the bacteria into heart-infusion broth. After 48 hr. aerobic incubation at 37°, the cultures were filtered. For each enzyme determination 3 ml. of the filtrate were used, and the estimations were carried out in the same way as those described for the proteinases of members of the *Clostridium* group.

Table III, which represents the activations and pH-optima of proteinases derived from *B. mycoides*, *Staph. citreus*, *Ps. fluorescens*, and *S. marcescens*, presents a uniform picture. The addition of cysteine, which activated the proteinases of the *Clostridium* group, produced in these cases a partial inhibition, while Fe<sup>++</sup> had an activating effect. Cysteine-Fe<sup>++</sup> gave the maximal activation. The mechanism of these activations, however, requires further investigation. The pH-optima of the investigated proteinases were at neutrality, which is in agreement with the results of Gorbach & Pirch [1936] and Maschmann [1937], who worked on the proteinase of *Ps. fluorescens*.

Table III. *Activation and pH-optimum of the proteinases of some aerobic bacteria, measured on gelatin*

		pH... 4	6	7	8
Activator		Activity in ml. of 0.05 N KOH			
<i>B. mycoides</i>	None	0.00	0.24	0.48	0.36
	Cysteine	0.00	0.12	0.28	0.14
	Fe <sup>++</sup>	0.00	0.40	0.65	0.49
	Cysteine-Fe <sup>++</sup>	0.00	0.48	0.75	0.54
<i>Staph. citreus</i>	None	0.00	0.78	1.12	0.64
	Cysteine	0.00	0.64	1.06	0.54
	Fe <sup>++</sup>	0.20	2.32	2.60	2.24
	Cysteine-Fe <sup>++</sup>	0.28	1.61	3.40	2.46
<i>Ps. fluorescens</i>	None	0.00	0.40	0.68	0.46
	Cysteine	0.00	0.16	0.37	0.25
	Fe <sup>++</sup>	0.11	0.80	1.21	0.78
	Cysteine-Fe <sup>++</sup>	0.10	0.88	1.23	0.86
<i>S. marcescens</i>	None	0.00	0.91	1.61	1.36
	Cysteine	0.00	0.64	1.16	0.48
	Fe <sup>++</sup>	0.00	1.61	2.23	1.82
	Cysteine-Fe <sup>++</sup>	0.08	1.69	2.52	2.08

The results given in Table IV, on the proteinase of *Pr. vulgaris*, resemble very much those obtained with the proteinases of the *Clostridia*. The difference between the activation behaviour of the proteinase of *Pr. vulgaris* and the proteinases of the aerobic organisms may be due to the fact that *Pr. vulgaris* is a facultative micro-organism. It is, however, quite possible that, by cultivating a facultative micro-organism for many generations aerobically, a proteinase may

Table IV. *Activation and pH-optimum of the proteinase of Pr. vulgaris*

Activator	pH... 4	6	7	8
	Activity of ml. of 0.05 N KOH			
None	0.00	0.41	0.75	0.48
Cysteine	0.00	0.40	0.86	0.49
Fe <sup>++</sup>	0.00	0.37	0.68	0.40
Cysteine-Fe <sup>++</sup>	0.00	0.99	1.56	1.13

be obtained which would have the characteristics of those obtained from the aerobic group of organisms. The possibility of such variability was demonstrated previously [Kocholaty & Weil, 1938].

### SUMMARY

Anaerobic organisms, including *Cl. histolyticum*, *Cl. sporogenes*, *Cl. Welchii*, *Cl. putrificum* and *Cl. botulinum*, secreted proteinases which were activated by cysteine. The combination of cysteine with Fe<sup>++</sup> gave the maximum activation.

The exocellular proteinase of *Cl. Welchii* hydrolysed clupein, gelatin, casein and Witte peptone.

Proteinases of certain aerobic organisms, *B. mycoides*, *Staph. citreus*, *Ps. fluorescens* and *S. marcescens*, were partially inhibited by cysteine. However, marked activations were effected by cysteine in combination with Fe<sup>++</sup>.

The proteinase of the facultative anaerobe, *Pr. vulgaris*, resembled the proteinases of the Clostridia in activation behaviour.

Optimal action at neutrality was observed in all the investigated bacterial proteinases.

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# CIX. THE COMPOSITION OF THE CROP MILK OF PIGEONS

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*(Received 5 April 1939)*

PIGEON squabs are hatched in the undeveloped state but grow very rapidly during the first few days after hatching. During this period they are fed on a special secretion formed in the crop of the parent birds. This secretion, commonly called "pigeon's milk", is a white, slimy, caseous material formed by the desquamation of epithelial cells and may be regarded as an example of a sacrifice of cell structure in the parent for the nutrition of the young. The endocrine control of its formation has been investigated by Riddle *et al.* [1932]. Its general composition and nutritive properties have been studied by Reed *et al.* [1932] and by Dabrowska [1932]. The former workers found the material to contain dry matter 35.7%, with protein 52.7, fat 35.6 and ash 4.5% of the dry matter; Dabrowska found dry matter 23.3%, and protein 57.4, fat 34.2 and ash 6.5% of the dry matter. The material did not contain carbohydrates.

In the present work, the compositions of four samples, pooled from 115 birds (three from 30 birds each, one from 25), have been investigated in detail. The birds had been used for prolactin assays, in which activity was measured by the wet weight of the crop glands. The gland contents were separated and made available for analysis as soon as possible after killing the birds.

## *Treatment of samples and methods of analysis*

The samples were examined for small pieces of broken grain which were removed by hand-picking and the wet samples were weighed. The samples were dried in dishes with intermittent stirring, first at 60° and finally at 100°, and the weight of dry matter was determined. The powdered material was exhaustively extracted in a Soxhlet extraction apparatus for 18 hr. with light petroleum (B.P. below 40°) and the solvent-free fat determined and used for analysis.

The residue was further extracted with a boiling mixture of 3 parts ethyl alcohol and 1 part benzene for 12 hr. and the solvent-free extract was weighed and used for analysis.

The dried residue was used for the proximate and detailed analysis of organic constituents and ash. No further purification of the residue was carried out before acid hydrolysis of the protein to determine the Hausmann nitrogen distribution, since the material contained 15% of nitrogen on a moisture and ash-free basis. The values of the humin nitrogen in all cases were satisfactorily low.

*Ash constituents.* The phosphorus and metallic radicles were determined on the ash by the usual standard methods, but chloride was determined on the original material by the open-Carius method, all results being calculated on the basis of the dry matter of the secretion. The compositions of the four samples are given in Table I.

*Protein.* The protein-rich, fat-free residues were hydrolysed (1 g. portions) with 20% HCl in an autoclave at 130° and the distribution of nitrogen in three groups was determined. The results are given in Table II.

Table I. *Proximate composition and detailed analysis of crop milk of pigeons*

Sample no.	I	II	III	IV	Lactose-free dried milk
No. of birds	30	25	30	30	—
Yield of wet material g.	37.7	10.6	29.4	43.4	—
Yield of dry material g.	10.54	5.61	8.49	12.35	—
Dry matter per bird g.	0.35	0.22	0.28	0.41	—
Dry matter %	27.9	53.1*	29.1	28.5	—
% of dry matter:					
Total lipoids	33.4	32.7	32.6	36.3	46.4
Ether-sol. lipoids	28.4	27.7	27.7	31.1	—
EtOH-C <sub>6</sub> H <sub>6</sub> -sol. lipoids	5.0	5.0	4.9	5.2	—
Nitrogen	9.38	9.47	9.54	9.10	—
Protein (N × 6.25)	58.6	59.2	59.6	56.9	42.9
Ash	4.81	4.45	4.41	4.76	10.7
Calcium	0.261	0.313	0.276	0.362	1.79
Potassium	0.976	0.957	1.022	1.028	2.00
Sodium	0.311	0.340	0.306	0.294	1.14
Phosphorus	0.987	1.039	1.009	1.037	1.43
Chloride	0.055	0.066	0.052	0.059	1.43
Starch	3.23	5.21	3.59	3.57	—
True protein as % of total protein	93.4	95.7	92.7	94.9	94.0
Total accounted for	99.0	101.6	100.2	101.5	—
Ca P (atom. equiv.)	3.8	3.3	3.7	2.9	1.0

\* Sample was partly dried before analysis.

Table II. *Analysis of protein of pigeon crop milk. Nitrogen distribution*

Sample no.	I	II	III	IV	
Protein in dry matter (N × 6.25) %	58.6	59.2	59.6	56.9	—
Nitrogen in ash- and fat-free dry matter %	14.78	14.74	15.15	15.33	—
% of total N:					Chicken muscle
Amide-N	9.2	7.7	7.8	6.6	7.5
Diamino-N	31.2	29.3	33.6	32.5	30.0
Monoamino-N	55.9	59.0	55.7	56.8	59.8
Humin-N	3.7	4.0	2.9	4.1	2.7

Table III. *Analysis of lipid extracts of pigeon crop milk*

Sample no.	I	II	III	IV
Total fat in wet matter %	12.0	—	11.2	12.7
Total fat in dry matter %	33.4	32.7	32.6	36.3
Light petroleum-extractable %	28.4	27.7	27.7	31.1
EtOH-C <sub>6</sub> H <sub>6</sub> -extractable %	5.0	5.0	4.9	5.2
Light petroleum extract:				
Refractive index (40°)	1.4642	1.4641	1.4642	1.4641
Saponification no.	181	180	181	182
Iodine value (Wijs)	60.5	60.3	62.4	61.3
Hegner number	93.5	92.8	93.8	93.0
Iodine value (fatty acids)	62.3	61.6	63.6	64.8
Phosphorus %	0.337	0.271	0.329	0.251
Nitrogen %	0.160	0.130	0.149	0.119
P/N (atomic)	0.95	0.94	1.00	0.95
Lecithin in extract %	8.7	7.0	8.5	6.5
EtOH-C <sub>6</sub> H <sub>6</sub> extract:				
Iodine value (fatty acids)	76.8	79.4	79.3	80.2
Hegner number	69.2	68.4	69.1	66.8
Phosphorus %	1.66	1.79	1.61	1.93
Lecithin in extract %	43.0	46.4	41.7	50.0

*Lipoids.* The largest yields of fatty material were obtained by the light petroleum extraction and these fractions were examined in detail for the characteristics of the fat. The examination of the second extraction with alcohol-benzene was carried out as far as the weight of extract permitted. Both samples were found to contain lecithin, the second being richer in it than the first. The amounts of lecithin were determined from the phosphorus contents of the fractions and the P/N ratio was determined in all samples. The analyses of the two lipid fractions are given in Table III.

*Food residues from the crop.* The presence of traces of food taken in by the parent birds was suspected in the samples. A microscopic examination of the dry matter after staining with aqueous I-KI revealed the presence of starch granules in all samples. The starch was determined by alcoholic precipitation after digestion with alcoholic KOH, hydrolysis with dilute HCl and oxidation with alkaline iodine. The values found varied from 3 to 5% and are given in Table I.

#### DISCUSSION OF RESULTS

The dry matter content of the secretion, except for the dried sample, averaged 28.5%, which is between the values given by other workers. The yield of dry matter per bird was very variable, namely, from 0.22 to 0.41 g.

The protein in the samples, at the degree of purification reached, contained from 14.8 to 15.3% of nitrogen. This value is somewhat low owing to the presence of starch. The average N content on a moisture-, ash- and starch-free basis, was 15.6%. Acid hydrolysis showed that the protein was in a fairly pure condition since the humin nitrogen did not exceed 4%.

The nitrogen distribution showed that the protein resembled closely chicken muscle protein. The content of diamino-acids was relatively low and that of the monoamino-acids consequently high. The composition of the protein, derived as it is from tissue protein, thus conformed more closely to that of muscle protein than to that of either egg albumin or blood serum proteins. The protein fraction contained roughly 6% of nitrogen in a non-protein form, or roughly the same amount as in blood serum and cow's milk. The composition of this fraction is probably the same as the "residual" fraction of blood and of other secretions.

The composition of the fat is unique in that it contains considerable quantities of lecithin. Some lecithin was directly extractable from the samples by light petroleum at 40° and a subsequent fraction extracted with an alcohol-benzene mixture was richer in lecithin. This pointed to the presence of lecithin in loose combination with protein in the material, namely, as a lecitho-protein. The behaviour of the lecithin during extraction was similar to that of the lecitho-protein of the protein residue obtained by Rewald [1939] from the extraction of butter fat from butter, in which the lecithin could be removed from protein combination by prolonged extraction with a hot alcohol-benzene mixture. The light petroleum extract contained on the average 7.7% of lecithin calculated from the phosphorus content of the extract, while the alcohol-benzene extract contained 45.3% of lecithin. Acetone precipitated the lecithin in the latter but not in the former fraction. The N/P ratio in the larger fraction was 0.96.

The fat on analysis yielded neither volatile fatty acids nor those of low molecular weight. The iodine value of the fats was 60.3-62.4, and of the fatty acids 61.6-64.8. The fat was of the same degree of unsaturation as goose-fat (58-62) but more saturated than hen fats (79-80) [cf. Hilditch *et al.* 1934]. The fatty acids of the alcohol-benzene fraction, however, showed a higher iodine value (77-80), which for a stearo-oleo-lecithin means 88% of oleic acid in the

fatty acid fraction. The Hehner number for lecithin is 70.5, but the values found for the second fraction were 67–69, which are considered low owing to loss during hydrolysis. The small amount of scum containing solid particles, which usually forms during the ether extraction of acidified, saponified extracts containing lecithin, formed in these cases also and was judged to contribute to the low values obtained for the Hehner number. The petroleum ether extracts behaved normally in this determination, and gave Hehner values comparable with those usually obtained for pure fats.

The mineral matter was characterized by its high K and P contents, medium Ca and low Na and Cl contents. The bulk of the ash consisted of potassium phosphate, as was shown by the readiness with which it fused. P/Ca ratios (atomic equivalents) were 3 or 4 : 1. Appreciable amounts of the P were present in the dried material in forms other than inorganic. Dilute acetic acid extracted only 60–70 % of the P in 16 hr., while a cold digestion for 16 hr. with 0.25 N KOH extracted only 64–73 %. There was no evidence of a phospho-protein present in the secretion.

Generally, the scheme of nutrition provided by the crop secretion favours muscular development, increase in blood volume and bone development. The scheme closely resembles that functioning in the egg before hatching, in that the secretion is devoid of carbohydrates, rich in fat, lecithin, P, K and protein, comparatively rich in Na and Ca and poor in chloride. The secretion bears some resemblance in composition to that of lactose-free dried cow's milk.

#### SUMMARY

Four samples of crop milk from 115 birds have been investigated in detail. The samples contained 28 % dry matter, with 33.8 % fat, 58.6 % protein, 4.6 % ash and 3.9 % starch (from an extraneous source) in the dry matter. The protein contained 94 % true protein; its nitrogen distribution resembled that of chicken muscle more closely than that of egg or serum proteins.

A light petroleum extract contained 7.7 %, and a subsequent alcohol-benzene extract 45.3 %, of lecithin, and it appeared as if the lecithin were in loose combination with the protein as lecitho-protein.

The ash was rich in K, Na, Ca and P, and about 30–40 % of the P was in organic combination. The secretion was poor in chloride.

The type of food furnished by the crop secretion is very similar to that provided by the egg before hatching.

The author is indebted to Dr S. J. Folley for placing the samples at his disposal.

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# CX. THE CARBAMIDO DIACETYL REACTION: A TEST FOR CITRULLINE

By WILLIAM ROBERT FEARON

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*(Received 6 April 1939)*

THE carbamido-acid citrulline, isolated by Koga & Odake in 1914 from the water melon, attracted no general attention until Wada [1930] established its constitution as  $\alpha$ -amino- $\delta$ -carbamidovaleric acid, and showed [1933, 1] that it also occurred among the products of the tryptic digestion of caseinogen.

The importance of the acid in nitrogen metabolism was revealed when Krebs & Henseleit [1932] demonstrated that citrulline is an intermediate in the mechanism whereby urea is formed in the liver.

These discoveries raise subsidiary questions regarding the distribution of citrulline in natural proteins, the origin and significance of carbamido-acids, and the nature of the mechanism involving the elaboration of citrulline from its precursors, ornithine, ammonia and carbon dioxide, and its subsequent conversion into arginine. An approach to the study of such problems is begun in the present paper by the introduction of a colour test by which citrulline can be detected in proteins, and its formation traced during a reaction cycle.

## *Colour reactions of urea and substituted ureas*

Since citrulline may be regarded as a substituted urea, a survey was made of urea colour tests applied to proteins. The most familiar of these tests depend on condensation between the urea and an aldehyde in strongly acid solution. The reagents employed include (i) furfuraldehyde [Schiff, 1877], (ii) furfuraldehyde and stannous chloride [Nakashima & Maruaka, 1923], (iii) methylfural [Fenton, 1903], (iv) *p*-dimethylaminobenzaldehyde [Barrenscheen, 1923].

Although these reagents yield colours with monosubstituted ureas, including citrulline, they are unsatisfactory as tests with most proteins, owing to the presence of tryptophan, which combines with the aldehyde to form pigments that are intensely coloured and completely obscure any reaction given by the citrulline.

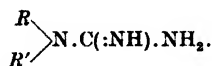
A study of the colour reactions between ketones and substituted ureas gave more promising results, for it was observed that diacetyl in strongly acid solution yielded products which, on careful oxidation, were converted into orange or red pigments. Under these conditions tryptophan gave no colour nor did the substituted guanidines, the latter observation indicating that the reactions of diacetyl in acid solution display a selectivity that differs from the reactions in alkaline solution, such as the familiar tests for arginine and creatine, discovered by Harden.

The scope and mechanism of the reactions of diacetyl in alkaline solutions have been investigated by Harden & Norris [1911], O'Meara [1931], and Lang [1932]. O'Meara (personal communication) has observed that diacetyl yields a yellow colour with urea in presence of conc.  $H_2SO_4$ , and Smith [1935] has described a similar series of colour reactions between diacetyl and many azides,

including semicarbazide. To avoid confusion, it is proposed to classify these various types of test as follows:

(1) *Carbamido diacetyl reactions*, which occur in acid solution, and are given by compounds containing the system  $R.NH.CO.NH_2$ , or, in certain instances,  $R_1.NH.CO.NH.R_2$ .

(2) *Guanidino diacetyl reactions*, which occur in alkaline solution, and are given by compounds containing the system  $R.NH.C(:NH).NH_2$ , or



While seeking a stable form of the diketone for use as a class reagent, both the monoxime and the dioxime were examined. The dioxime, as might be expected from its greater stability, was unsatisfactory, but the monoxime proved to be superior in range and sensitivity to ordinary diacetyl. For example, symmetrical dimethylurea yields a deep purple colour with the monoxime reagent, but very little colour with diacetyl itself. In applying the test to substituted ureas, the solution is strongly acidified with HCl, a few drops of 3% diacetyl monoxime are added, and the mixture is boiled for 3–4 min., during which time an orange colour develops if the test be positive. The colour deepens to red on standing or after addition of a drop of 1% potassium persulphate.

#### *The scope of the carbamido diacetyl reaction*

Using diacetyl monoxime, followed by an oxidizer, the test is positive with: urea, methylurea, butylurea, phenylurea,  $\beta$ -naphthylurea, dimethylurea (symmetrical and asymmetrical), allantoin, semicarbazide, citrulline and all higher proteins examined (ovalbumin, serum albumin, seroglobulin, caseinogen, lactalbumin, fibrin, edestin, gluten, mucin). Commercial peptones and gelatins also give the reaction with varying degrees of intensity.

The test is negative with: ammonium salts, hydrazine, carbamate, cyanate, acetamide, acetylurea, diphenylurea (asymmetric), guanidine, methylguanidine, creatine, creatinine, glycoeyamine, uroxamic acid, uric acid, indole and all amino-acids examined, other than citrulline (glycine, glycine ester, sarcosine, alanine, cystine, tyrosine, tryptophan, arginine, histidine, lysine, proline, hydroxyproline, asparagine, aspartic acid).

*Reaction with urea.* Urea is the only compound so far observed which yields a bright yellow colour, deepening to orange on oxidation. The test is very sensitive, and will reveal 0.1 mg. of urea in 2 ml. of solution, and may be used to show the presence of urea as a contaminant in the monosubstituted ureas, which give a red colour with the reagent. With semicarbazide, the reaction is exceptional in that pigment formation proceeds rapidly in the absence of an external oxidizer, and the compound gradually separates out as a dark red precipitate. Conversely, semicarbazide provides a useful reagent for the detection of diacetyl in aqueous extracts of butter and similar materials.

*Reaction with citrulline.* The test will give a well-marked red colour with citrulline in concentrations of  $M/100$  (0.175%), and will detect the carbamido-acid in concentrations as low as 0.01%. The colour changes from Madeira to garnet red during oxidation and closely matches the colour given by a tryptic digest of caseinogen, but is redder than the colour given by unhydrolysed caseinogen. It can be matched within ordinary limits by the colour given by pure methylurea or butylurea, uncontaminated by ordinary urea.



*The carbamido diacetyl reaction with proteins*

About 2 ml. of the protein solution are treated with at least twice as much conc. HCl and 3-5 drops of 3% aqueous diacetyl monoxime. The mixture is boiled for 30 sec., allowed to cool for 2 min., and then carefully oxidized by addition of 1-3 drops of dilute (1%) potassium persulphate or very dilute (0.01%) hydrogen peroxide. A carmine colour develops if citrulline or a similar carbamido-compound be present. The colour deepens on standing or on warming gently. A slight excess of the oxidizer rapidly bleaches the pigment in hot solution.

*Notes on the test.* (1)  $\text{H}_2\text{SO}_4$ ,  $\text{H}_3\text{PO}_4$  or trichloroacetic acid may be used as condensing agents instead of HCl, but appear to offer no advantages. By adding conc.  $\text{H}_2\text{SO}_4$  to a mixture of protein and diacetyl monoxime the reaction may be obtained as a "ring" test. In this form it is slow, and several hours are required for colour development at room temperature.

(2) The quantity of acid used should be sufficient to redissolve any protein precipitated by the first addition of acid, previous to heating the mixture.

(3) For maximum colour development, oxidation must be carefully controlled. While almost any of the commoner oxidizing agents may be used, persulphate appears to be the least destructive to the formed pigment. The reaction is inhibited by copper salts, and for this reason is not given by copper citrullinate.

(4) The time of boiling must be curtailed when dealing with proteins rich in carbohydrate residues but relatively poor in citrulline, such as the albumins, or protein preparations containing carbohydrate, such as dried milk powder. When boiled with excess of acid, furfuraldehydes are liberated from the carbohydrate and react with tryptophan in the protein to form violet pigments (Liebermann-Cole reaction). The effect of this accompanying reaction is to change the tint of the citrulline colour from red to purple. Furfuraldehyde pigment formation can be suppressed by avoiding prolonged boiling and the use of excess of protein. Where there is uncertainty, a control test should be carried out in which the diacetyl reagent is omitted. After oxidation, the control test may develop a lilac colour, but neither the intensity nor the shade is comparable with the citrulline colour. When dealing with materials free from tryptophan, such as gelatin, boiling may be continued for several minutes, and the colour allowed to develop spontaneously by atmospheric oxidation. Samples of crude caseinogen prepared from sour milk may contain diacetyl or its precursor, acetylmethylcarbinol (acetoin) in quantities sufficient to give a distinct carbamido-test in the absence of the reagent.

(5) Caseinogen and egg albumin in 1% solutions give a bright purple-red colour with the reagent. Gelatin in 1% solution gives a very feeble reaction unless boiling be continued for at least 5 min. As none of these colours exactly matched the colour given by free citrulline, an endeavour was made to determine approximately the citrulline content of caseinogen by applying the test to a 10% solution that had been subjected to prolonged tryptic digestion. After treatment with charcoal, 1 ml. samples of the digest were submitted to the carbamido-test and the colours compared with that given by a standard ( $M/100$ ) solution of citrulline. Good comparisons were obtained, the colorimetric readings indicating a citrulline value for the caseinogen of the order of 2.0-2.5%.

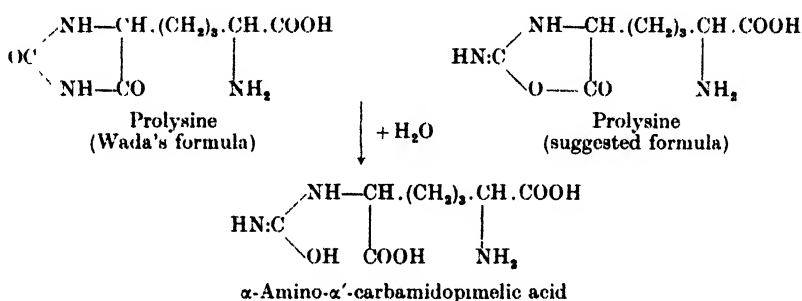
*The significance of the carbamido diacetyl reaction with proteins*

Citrulline is readily obtained from arginine by alkaline hydrolysis or by bacterial degradation [Ackermann, 1931; Horn, 1933], and its appearance in tryptic digests does not necessarily indicate that it is a preformed component of the original protein molecule, although Wada has claimed that citrulline may be isolated from hydrolysed caseinogen under conditions that do not lead to the conversion of arginine into the carbamido-acid. The present test, it is believed,

affords direct evidence for the existence of citrulline in natural proteins; nevertheless, several possible sources of error must be considered, especially when dealing with proteins hydrolysed in alkaline solution. Some preparations of trypsin, notably commercial pancreatic extracts and autolysed pancreas, are very rich in material giving a positive carbamido-reaction indistinguishable from citrulline, and thus may contribute to the citrulline value of a digest.

Free arginine is readily hydrolysed in alkaline solution (e.g.  $N/2$  NaOH) at room temperature, and after a few days yields a gradually intensifying series of carbamido-reactions, and the citrulline value of an apparently sterile tryptic protein digest may increase considerably with time. Lastly, carbamido-acids other than citrulline may be present in the original protein or may arise during hydrolysis. Prolysine, discovered by Wada [1933, 2], is rapidly decomposed by weak alkalis with production of  $\alpha$ -amino- $\alpha'$ -carbamidopimelic acid, which gives a positive carbamido-reaction.

Indeed, the instability of prolysine in presence of alkalis is such that one is led to question Wada's formula, and regard the compound as containing an ester linkage rather than the conventional ureide structure.

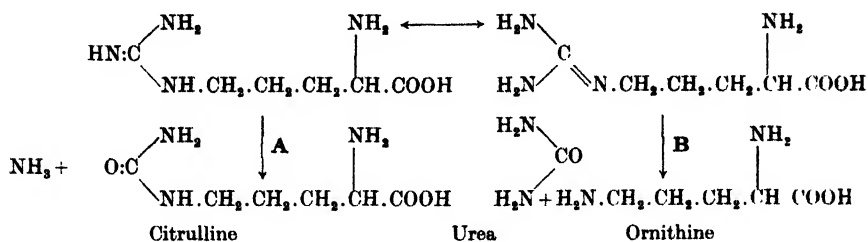


Prolysine, however, is much more stable in acid solutions, and is unlikely to cause a fallacy when the carbamido-test is applied to intact proteins.

#### *A note on the hydrolysis of arginine*

The citrulline required for the present work was prepared from arginine by alkaline hydrolysis, according to the method of Fox [1938]. The arginine used was the purest carbonate obtainable from Messrs Schuchardt, and gave no reaction with the carbamido-test. The citrulline was isolated as the copper salt, and was subsequently recrystallized twice. The crystalline carbamido-acid began to melt at  $218^\circ$  and was completely melted at  $223^\circ$  (uncorrected). During trial preparations of citrulline some observations were made on the course of arginine hydrolysis in  $0.1 N$  and  $5 N$  NaOH. The mixtures were boiled under a reflux condenser fitted to an aspirator and acid trap to retain the liberated ammonia. Within the first 15 min. citrulline could be detected by means of the carbamido-test in samples from the mixture. Urea accompanied the citrulline, as was suggested by the colour of the carbamido-reaction, which was yellower in shade than that given by pure citrulline, and the presence of urea was confirmed by the urease test. After 4 hr. hydrolysis in presence of  $5 N$  NaOH, the mixture contained in addition cyanate, derived no doubt from the decomposition of urea, and also tetramethylenediamine, derived from the ornithine.

Since urea is not formed from citrulline under these conditions of hydrolysis, it must arise from the arginine directly, the decomposition of which can be represented as following two independent paths.



Reaction A is catalysed by the arginine desimidase, described by Horn [1933]. Reaction B is catalysed by arginase. During alkaline hydrolysis both reactions occur, but the conditions that determine their relative extents have not yet been elucidated; increase in the concentration of alkali promotes reaction A. For simplicity, the compounds are not represented as zwitterions or as resonance hybrids in the above formulation.

#### *Chemistry of the carbamido-diacetyl reaction*

From inspection of the formulae of the reactants, it appears that the test is positive with compounds containing the system  $R_1\text{.NH.CO.NH.R}_2$ , where  $R_1$  is either hydrogen or a simple aliphatic radicle, and  $R_2$  is not an acyl radicle. Thus, phenylurea and symmetrical dimethylurea both form pigments, whereas acetylurea and symmetrical diphenylurea do not. In the absence of an oxidizing agent, diacetyl unites with substituted ureas in acid solution to form cyclic derivatives or diureins investigated by Biltz [1907]. These compounds are colourless and do not yield pigments on oxidation. Their formation involves both ketone groups of the diacetyl. It is suggested that the pigments obtained in the carbamido-reaction are derivatives of a pyrimidine ring, the closure of which is brought about by oxidative condensation involving one methyl group of the diacetyl. The compound obtained from diacetyl and semicarbazide has already been obtained by Thiele [1898], and shown to be a triazine derivative.

#### SUMMARY

A colour test is described for citrulline and other substituted ureas. The test is capable of being applied directly to proteins, and affords evidence that citrulline exists preformed in caseinogen, egg albumin, and other natural proteins.

Mr E. Kawerau has assisted me in the preparation of the citrulline used in the present work, and I am indebted to Prof. E. A. Werner for specimens of various substituted ureas.

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# CXI. IMMUNOLOGICAL AND CHEMICAL PROPERTIES OF CARBOBENZYLOXY- PROTEINS<sup>1</sup>

## I. SERUM GLOBULIN AND EGG ALBUMIN DERIVATIVES

## II. INSULIN DERIVATIVES

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IN previous communications [Hopkins & Wormall, 1933, 1, 2; 1934, 1] the chemical and immunological properties of phenyl- and *p*-bromophenyl-carbamido protein compounds have been described. These compounds were obtained by the condensation of phenyl- and *p*-bromophenyl-isocyanates with various proteins. In other investigations the action of these arylisocyanates on insulin has been studied [Hopkins & Wormall, 1934, 2; Gaunt & Wormall, 1936; cf. also Jensen & Evans, 1934; 1935].

Bergmann and his colleagues [Bergmann & Zervas, 1932; cf. review by Bergmann, 1933] have shown that benzyl chloroformate readily reacts with the amino group of amino-acids in cooled alkaline solution yielding carbobenzyloxy-amino-acids from which the amino-acid can be regenerated by catalytic reduction with hydrogen in the presence of palladium black. There is no direct evidence that benzyl chloroformate reacts with native proteins in this way, i.e. by condensation with the free amino-groups of the protein molecule: it was thought, however, that if such a reaction occurred, it would be suitable for the investigation of the immunological properties of proteins and also the chemistry of insulin. Preliminary experiments were therefore carried out to see whether benzyl chloroformate reacts with serum globulin, egg albumin and insulin. Bergmann's method was modified slightly, the solution being maintained at pH 7.5-8.5 during the reaction in order to minimize the possibility of other changes in the protein molecule taking place. As is described below, this treatment with benzyl chloroformate converts antigenic proteins into acid-precipitable derivatives which have a new antigenic specificity, and in the case of insulin produces a derivative which is devoid of hypoglycaemic power. It was also thought that the new grouping introduced into the protein molecule might be removed by catalytic reduction, with, perhaps, the reproduction of the original immunological specificity in the case of the native proteins and the regeneration of the hypoglycaemic activity in the case of insulin.

<sup>1</sup> The investigations described here were mainly carried out when the authors were members of the Department of Physiology, the University of Leeds.

In the description below, the investigation is divided into two parts, the first part dealing with the preparation and the chemical and immunological properties of carbobenzyloxy-protein compounds, and the second part with the inactivation of insulin by benzyl chloroformate and the chemical and immunological properties of carbobenzyloxy-insulin.

## I. SERUM GLOBULIN AND EGG ALBUMIN DERIVATIVES

### *Preparation of compounds*

*Carbobenzyloxy-horse serum globulin.* The serum globulins were separated from horse serum by means of  $\text{CO}_2$  [Hopkins & Wormall, 1933, 1]. 500 ml. of a 2.4% globulin solution were mixed with 400 ml. of phosphate buffer of pH 8. The mixture was cooled in ice and 12 ml. of benzyl chloroformate in 50 ml. of ether added slowly to the well-stirred solution. The reaction was maintained at about pH 8 by addition of 2N NaOH. The globulin compound slowly precipitated out and after 3 hr. the product was centrifuged. The supernatant solution contained no protein and was therefore rejected. The solid material was suspended in water at pH 7.5 and extracted 5 times with 30 ml. of ether to remove the excess of benzyl chloroformate. The ether remaining in the suspension was removed by evacuation. The protein material was washed twice with 400 ml. of 0.9% NaCl solution, and the reaction adjusted to pH 8.0. Finally the carbobenzyloxy-horse serum globulin was ground in a mortar, strained through fine muslin and suspended in 200 ml. of 0.9% NaCl, the reaction being adjusted to pH 7.5.

*Carbobenzyloxy-gelatin.* 10 g. of gelatin (Coignet's "Gold Label") were dissolved in 300 ml. of water, and 600 ml. of phosphate buffer pH 8 and 900 ml. of water were added. The mixture was cooled, 7.5 ml. of benzyl chloroformate in 100 ml. of ether added slowly and the whole mixture was stirred for 3 hr. at room temperature, the reaction being maintained at pH 8. At the end of 3 hr. the mixture was centrifuged and the supernatant liquid cooled and acidified with 2N HCl to give maximum precipitation of the carbobenzyloxy-gelatin. This product was separated by centrifuging, dissolved in water at pH 8.5 by gentle warming to 36–37° and reprecipitated by HCl. After a third precipitation the product was dissolved in the minimum amount of water (180 ml.) and the pH adjusted to 7.5.

*Carbobenzyloxy-egg albumin.* 200 ml. of a 1.7% solution of crystalline egg albumin [Adair & Robinson, 1930] were treated as above with 200 ml. of phosphate buffer of pH 8 and 7.5 g. of benzyl chloroformate in 30 ml. of ether. The mixture was stirred at 5° for 3 hr. and then centrifuged. The supernatant solution was acidified with dilute acetic acid to give maximum precipitation of the carbobenzyloxy-egg albumin. This protein derivative was then separated by centrifuging and redissolved in water by adding 2N NaOH to give pH 8. The solution was centrifuged and the protein derivative in the supernatant solution was precipitated by dilute HCl. After two further precipitations the carbobenzyloxy-egg albumin was dissolved in 0.9% NaCl at pH 7.5. A small amount of chloroform was added as a preservative to the solutions of the carbobenzyloxy derivatives of gelatin and egg albumin.

*Carbobenzyloxy-amino-acids.* For the purposes of inhibition tests a few carbobenzyloxy-amino-acids were made. The method described by Bergmann &

Zervas [1932] for the synthesis of peptides has been used so widely that the carbobenzyloxy-derivatives of most of the naturally occurring amino-acids have been prepared, usually by addition of benzyl chloroformate to a cooled aqueous solution of the amino-acid in the presence of a considerable excess of alkali. For the purposes of the present research it was thought advisable to reproduce in these preparations the conditions obtaining in the production of the carbobenzyloxy-protein derivatives, i.e. to carry out the reaction in the presence of phosphate buffer at pH 8.

#### *Immunization*

The carbobenzyloxy-horse serum globulin was prepared as described above and the suspension kept in the ice chest, phenol being added to give a concentration of 0.25% in the suspension. The suspension was injected intraperitoneally into four rabbits at intervals of 7–9 days, each rabbit receiving at each injection a suspension containing 0.25 g. of the protein. The sera were tested 7 days after the third and fifth injections. One rabbit gave a good antiserum after three injections; the others required two further injections.

#### *Precipitin tests*

Precipitin tests were carried out as previously described [Hopkins & Wormall, 1933, 1]. Readings were taken after  $\frac{1}{2}$ , 1 and 2 hr. at 37° and usually after a further 18 hr. at room temperature; those recorded in Tables I and II were obtained after 1 hr. In the case of carbobenzyloxy-gelatin compounds the tests were made at room temperature (15–19°), more pronounced precipitates being obtained in this way [Hopkins & Wormall, 1933, 2].

The extent of precipitin formation is recorded as follows (in increasing degrees of precipitation):

— (no reaction), f.tr. (faint trace), tr. (trace),  $\pm$ , +,  $+\pm$ , ++.

#### *Inhibition tests*

Precipitin inhibition tests were carried out under the conditions previously described [Hopkins & Wormall, 1934, 1], the inhibiting substance being in neutralized 0.01 *M* solution. The degree of precipitin formation was noted immediately and after 5 min.,  $\frac{1}{2}$  and 1 hr. These inhibition tests were carried out at room temperature when carbobenzyloxy-gelatin was used as the antigen, and at 37° in other cases.

#### *The immunological properties of carbobenzyloxy-proteins*

The sera obtained by the injection of carbobenzyloxy-horse serum globulin into rabbits were tested against horse serum, carbobenzyloxy-egg albumin and carbobenzyloxy-gelatin. Marked reactions were obtained with the last two antigens (cf. Table I), indicating the possession of good antigenic power by the carbobenzyloxy-globulin. With horse serum the reactions were either feeble or negative, indicating almost complete loss of the original species specificity. The precipitin reactions with carbobenzyloxy-egg albumin and carbobenzyloxy-gelatin show that an immunological relationship exists between carbobenzyloxy-horse serum globulin and the corresponding derivatives of widely differing proteins. This relationship has been undoubtedly conferred on these proteins (e.g. gelatin and egg albumin) by the introduction of the carbobenzyloxy-grouping. The reactions with the gelatin compound are more strongly marked than those with the egg albumin compound. This is particularly interesting since gelatin itself is non-antigenic, whereas egg albumin is a true antigen.

In order to obtain confirmation of the view that the reaction between benzyl chloroformate and proteins involves the free amino groups of the protein molecule a few inhibition tests have been carried out. The results given in Table II show that carbobenzyloxy-glycine inhibits the reaction between carbobenzyloxy-proteins and the antisera to carbobenzyloxy-horse serum globulin, whereas it has no influence at all on the formation of precipitates in other antigen-antibody reactions (e.g. the horse serum anti-horse serum precipitin reaction). The carbobenzyloxy-protein precipitin reaction is partially inhibited by phenyl-carbamido-glycine.

Table I. *Precipitin reactions between carbobenzyloxy-protein derivatives and antisera to carbobenzyloxy-horse serum globulin*

Antigen	Dilution of antigen	Immune serum against carbobenzyloxy-horse serum globulin		
		No. 420	No. 421	No. 422
Horse serum	1:20	tr.	—	f.tr.
	1:100	tr.	tr.	f.tr.
	1:500	±	tr.	±
	1:2500	tr.	—	tr.
Carbobenzyloxy-egg albumin	1:20	f.tr.	—	f.tr.
	1:100	±	+	tr.
	1:500	±	+	±
	1:2500	tr.	±	±
Carbobenzyloxy-gelatin	1:20	tr.	f.tr.	tr.
	1:100	+	+	+
	1:500	+ ±	+ ±	+ ±
	1:2500	tr.	±	±

The dilutions of antigen refer to dilution of a solution which contains 5% of total protein.

Table II. *Inhibition of the precipitin reactions*

Antigen	Antiserum against	NaCl	"Inhibiting" substance		
			Glycine or alanine	Carbobenzyloxy-glycine (or alanine)	Phenyl-carbamido-glycine
Horse serum	Horse serum	++	++	++	++
Carbobenzyloxy-gelatin	Carbobenzyloxy-horse serum globulin	(a) + ±	+ ±	—	+
		(b) +	+	—	±

(a) and (b) give the results with different antisera.

## II. INSULIN DERIVATIVES

The effect on insulin of chemical reagents which possess a more or less specific action on certain groups present in proteins has been studied by many authors. By these methods a considerable amount of information has been obtained regarding the groupings in the insulin molecule which may be responsible for its hypoglycaemic activity. A few of these methods which result in the inactivation of the hormone are reversible, i.e. the activity of the original insulin can, at least in part, be regenerated under suitable conditions. Partial reactivation can be effected in insulin inactivated by acetic anhydride, formaldehyde or hot dilute acid [for a review of the literature cf. Freudenberg *et al.* 1930; 1931; Jensen & Evans, 1934], by acid-alcohol [Carr *et al.* 1929; Charles & Scott, 1931],



and by mild reducing agents such as cysteine and glutathione [Freudenberg & Wegmann, 1935]. Perhaps the most satisfactory reactivation of this type is, however, that obtained by Harington & Neuberger [1936]. These authors have shown that iodination of insulin, resulting in the substitution of iodine in the 3:5-positions of the tyrosine groupings, causes a 90–95 % loss of activity. Partial removal of the iodine by catalytic reduction is accompanied by an approximately proportional restoration of the hypoglycaemic activity.

For the reasons stated in the first part of this paper, it seemed likely that benzyl chloroformate would combine with the insulin molecule, possibly with loss of the hypoglycaemic activity of the hormone. Furthermore, it was thought that, if inactivation occurred, it might be found to be reversible, since the carbobenzyloxy-groupings attached to amino-acids can be removed by catalytic reduction. Preliminary experiments showed that benzyl chloroformate reacts with insulin and the properties of the product of this reaction (carbobenzyloxy-insulin) have been studied. A brief report of these investigations has been made [Gaunt *et al.* 1935].

#### EXPERIMENTAL

The insulin activities of the original material and the treated insulin were determined as previously described [Hopkins & Wormall, 1934, 2]. In practically all the experiments, tests were made with groups containing four rabbits. Duplicate sugar determinations (0.2 ml. blood for each) were carried out with every sample of blood from each rabbit, the method of Hagedorn & Jensen [1923] being used. In the tables the average value for each group is given.

The reaction between benzyl chloroformate and insulin was allowed to proceed at 5–8° and in the presence of 0.2 *M* NaHCO<sub>3</sub> at pH 8–8.5. The use of bicarbonate instead of the phosphate buffer of pH 8 is the only modification of the method employed in the experiments with native proteins as described in the previous part of this paper. The insulin used for this work contained 19,500 units per g.

In all experiments a portion of the insulin solution was removed, before any treatment, for use as a control. This control was usually subjected to the same treatment as the experimental sample without, of course, the addition of benzyl chloroformate. The various solutions injected into the rabbits were treated with phenol (to give 0.25 %).

#### *The action of benzyl chloroformate on insulin*

32.8 mg. of insulin were dissolved in 4 ml. of 0.9 % NaCl and 4 ml. of 0.2 *M* NaHCO<sub>3</sub>, 2 drops of 0.2 *N* NaOH being added to give a clear solution at pH 8–9; 0.5 ml. of this solution was removed for use as a control in the physiological assay. The remainder of the insulin solution was cooled in ice and treated with 0.8 ml. of benzyl chloroformate, the reaction being maintained at pH 8 by addition of 0.2 *N* NaOH when necessary. After the mixture had been shaken for 2 hr., the excess of benzyl chloroformate was removed by shaking eight times with 10 ml. ether at each extraction. The residual ether was removed by gentle warming and evacuation. The suspension of carbobenzyloxy-insulin was injected in such amounts that each rabbit received per kg. the treated material from 5 to 15 units of insulin. From the results of the blood sugar determinations it could be concluded that probably all the activity of the insulin had been destroyed as a result of treatment with benzyl chloroformate (cf. Table III).

As the insulin compound is insoluble it was thought advisable to determine whether the inability of carbobenzyloxy-insulin to lower the blood sugar of starved rabbits was due simply to its insolubility. If this is true, then the

administration of large amounts of insulin compound might exert a slow effect after injection. Blood sugar determinations carried out up to about 24 hr. after the injection indicate, however, that the carbobenzyl-oxy-insulin preparations have no power to reduce the blood sugar even when observations are made over a long period of time.

Table III. *Inactivation of insulin by benzyl chloroformate*

Group	Subcutaneous injection of	No. of units per kg.	Blood sugar (mg./100 ml.) Time after injection (hr.)				
			0	2½	4½	5½	23½
A	Insulin	0.75	82	49	55	70	107
B	Carbobenzyl-oxy-insulin	15	91	97	99	106	96

*The rate of inactivation of insulin by benzyl chloroformate*

Experiments have been carried out to determine the rapidity with which benzyl chloroformate inactivates insulin. A solution of 41 mg. of insulin in 0.9% NaCl and 0.2 M NaHCO<sub>3</sub> was cooled in ice and treated with 0.5 ml. of benzyl chloroformate, the mixture being well shaken. Samples were removed at intervals of 5, 30 and 60 min. These samples, and the control sample removed before addition of the reagent, were made slightly acid and immediately extracted six times with about 10 ml. portions of ether. The remaining ether was removed by gentle warming and evacuation. The 5, 30 and 60 min. samples, injected into rabbits in amounts equivalent to 4, 10 and 15 units of the original insulin respectively, showed no significant insulin activity (cf. Table IV). The results of this experiment indicate that the inactivation of insulin by benzyl chloroformate is rapid, though it is probable that the results obtained are only of qualitative value, as it is difficult to ensure complete removal of the excess of benzyl chloroformate by extraction with ether.

Table IV. *Time required for inactivation of insulin by benzyl chloroformate*

Preparation	No. of units per kg.	Blood sugar (mg./100 ml.) Time after injection (hr.)				
		0	1½	3½	5½	
Insulin	0.75	103	50	46	47	
Carbobenzyloxy-insulin	5 min.*	4	103	99	94	97
	30 min.*	10	104	101	98	96
	60 min.*	15	94	103	100	101

\* These samples were obtained by allowing the benzyl chloroformate to act on the insulin for 5, 30 and 60 min. (see text for full details).

*The effect of small amounts of benzyl chloroformate on insulin*

It is possible that total inactivation of insulin may occur when only a few of the free amino-groups of the insulin molecule have been blocked by benzyl chloroformate. In order to test this hypothesis experiments were carried out to determine the minimum amount of benzyl chloroformate required to effect this inactivation. For the purposes of these experiments the amounts are expressed in terms of that needed to combine with all the amino-groups of the insulin molecule, based on the assumption that insulin contains 1% of amino-nitrogen.

In the first experiment it was found that 12.5 times the theoretical amount of benzyl chloroformate inactivated insulin completely. In a second experiment of a similar type approximately 5, 3 and 2 times the theoretical amounts of

benzyl chloroformate were allowed to react with insulin. The blood sugar values obtained after the injection of the products of these reactions into starved rabbits are given in Table V. From these figures it will be seen that 5 and 3 times the theoretical amounts of benzyl chloroformate cause complete inactivation of insulin, and twice the theoretical amount destroys at least 95 % of the original hypoglycaemic activity of the hormone.

Further experiments were carried out using the theoretical and half-theoretical amounts of benzyl chloroformate. The results indicate that with the theoretical quantity about 60 % of the original activity is retained, whilst with half the theoretical amount of the reagent over 80 % of this activity remains (Table VI).

Table V. *Effect of different amounts of benzyl chloroformate on insulin*

Amount of benzyl chloroformate allowed to react with insulin	Amount of product injected ("units" per kg.)	Blood sugar (mg./100 ml.) Time after injection (hr.)		
		0	1½	4½
0	0.75	103	57	88
2 × theoretical	2.75	94	92	85
2 ×	0.75	91	97	104
3 ×	0.75	91	100	107
5 ×	1.5	95	100	109

*Experimental details.* 32.5 mg. of insulin were dissolved in 4 ml. of 0.9 % NaCl and 4 ml. of 0.2 M NaHCO<sub>3</sub> at pH 8-8.5. The solution was divided into 4 lots each of 2 ml., all the samples being then cooled in ice. To three of these samples were added 0.96 ml., 0.58 ml. and 0.38 ml. of a 0.5 % solution of benzyl chloroformate in ether. The volume of ether in all four samples was made up to 0.96 ml. The samples were shaken for 2 hr. and then left overnight in the ice chest. The ether was removed by warming and evacuation. The pH of the solutions did not alter during the course of the reaction. After suitable dilution with 0.9 % NaCl the samples were injected into rabbits.

Table VI

Amount of benzyl chloroformate allowed to react with insulin	Amount injected ("units" per kg.)	Blood sugar (mg./100 ml.) Time after injection (hr.)		
		0	1½	6
0	0.6	105	51	68
0	0.3	99	69	97
Theoretical amount	0.6	96	66	78
Theoretical amount	0.3	92	67	78
0.5 × theoretical amount	0.6	88	45	55
0.5 × theoretical amount	0.3	86	62	78

*Experimental details.* Similar to those for Table V, with smaller amounts of benzyl chloroformate.

#### *Attempts to reduce carbobenzyloxy-proteins catalytically*

In view of the ease with which the carbobenzyloxy-group can be removed from carbobenzyloxy-amino-acids by catalytic reduction [Bergmann & Zervas, 1932], it was decided to attempt a similar reduction with the carbobenzyloxy-derivatives of insulin and other proteins. Preliminary experiments were first made to determine the effect of catalytic reduction on untreated insulin. Treatment with hydrogen in the presence of palladium black was found to effect in some experiments, but not in all, appreciable inactivation of insulin. The palladium black undoubtedly adsorbs some of the insulin, but this adsorption (measured by total N determinations on the solutions) was not always sufficient to account for the loss of activity. Freudenberg *et al.* [1931] also experienced this adsorption in their efforts to reduce insulin catalytically, and were unable to overcome the difficulty. Since the experiments described here were completed, Harington & Neuberg [1936] have effected the removal of about two-thirds of

the iodine of iodinated insulin by catalytic reduction with a palladium-barium sulphate catalyst in pyridine-water solution and an atmosphere of hydrogen; this reduction does not appear to have any destructive action on the physiologically active groups of insulin.

In view of the difficulties experienced with insulin, further experiments were made with carbobenzyloxy-gelatin. No significant increase in free amino-N occurred, however, when this derivative was exposed in solution at pH 7 to the action of palladium black and hydrogen for several hours. It was concluded that some other method would have to be devised for the removal of the carbobenzyloxy-groups and in the case of the insulin derivative care would have to be taken to prevent loss of physiological activity due to reduction of the S—S groups.

Since the work described in this paper was completed, Clutton *et al.* [1937] and Clutton *et al.* [1938] have succeeded in removing the carbobenzyloxy-grouping from *O*- $\beta$ -glucosido-*N*-carbobenzyloxytyrosyl protein compounds. In the case of the gelatin compound it was found impossible to effect this removal by reduction in aqueous solution with a variety of catalysts and reducing agents. However, by reduction with sodium in liquid ammonia the carbobenzyloxy-grouping was removed from the *O*- $\beta$ -glucosido-gelatin derivative without rupture of the glucosido-linkage and without degradation of the protein. This method, however, could not be used in the case of *O*- $\beta$ -glucosido-*N*-carbobenzyloxytyrosyl horse serum globulin; here removal of the carbobenzyloxy-grouping was effected by reduction at pH 9.6 in an atmosphere of hydrogen in the presence of palladium black. These workers did not attempt to remove the carbobenzyloxy-grouping from *O*- $\beta$ -glucosido-*N*-carbobenzyloxytyrosyl insulin and little information is still available regarding the influence of such reducing agents on the insulin molecule.

Numerous authors have observed allergic reactions following the injection of insulin into man, and although in most instances the reactions observed can be attributed to other proteins present in the insulin preparations, there is evidence that pure insulin occasionally produces an allergic response. It is not certain that this hypersensitivity is due to a true antigen-antibody reaction, but the blood of hypersensitive patients has been shown to contain precipitins to insulin [Raynaud & LaCroix, 1925; Karr *et al.* 1933]. Stronger evidence in support of the view that insulin can function as an antigen has been obtained by Lewis [1937]. This author injected relatively large doses of insulin into virgin guinea-pigs, controlling the hypoglycaemia by the administration of glucose, and found that the uteri of these sensitized animals reacted with insulin but not with the pancreas protein or with the serum of the animal from which the insulin was derived. Cross-reactions with hog and beef insulins indicated that insulins from different animal species are immunologically closely related.

The injection of large amounts of insulin into an animal for the production of antibodies is precluded by the intense hypoglycaemic effect of the hormone, although the hypoglycaemia can be partially controlled by the administration of glucose. An alternative method is to inactivate those groups which are responsible for the hypoglycaemic activity by some process which does not destroy the antigenic power of proteins; this inactivated product could then be injected in amounts which are normally needed to incite antibody response to antigenic proteins. From evidence produced in this paper benzyl chloroformate appears to be suitable for this purpose, since it does not appreciably reduce the antigenic power of serum globulins and yet it rapidly destroys the hypoglycaemic activity of insulin. An experiment has therefore been carried out to determine whether

the injection of large amounts of carbobenzyloxy-insulin produces in mice any change in the sensitivity of the animals towards insulin, and whether any serological evidence of the formation of an anti-insulin can be obtained.

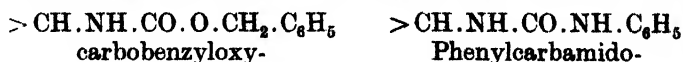
A group of 40 mice was given a series of injections of carbobenzyloxy-insulin and the animals subsequently tested for their response to insulin, and their serum for antibodies to carbobenzyloxy-insulin.

0.4 g. of insulin was treated as described previously with five times the "theoretical" amount of benzyl chloroformate, and the final product diluted to 56 ml. with the addition of NaCl (to give 0.9%) and phenol (0.2%); 0.25 ml. of this solution contained the equivalent of 1.8 mg. of insulin. Each mouse received four intraperitoneal injections, each of 0.0125 ml. of this solution per g., at intervals of 2-3 days. During the period of immunization the mice were fed on bread soaked in 10% glucose solution and were given a strong solution of glucose to drink in order to counteract the slight hypoglycaemic action of the carbobenzyloxy-insulin. Any mouse which showed signs of hypoglycaemia was given a subcutaneous injection of 0.25-0.5 ml. of sterile 10% glucose, but in spite of this treatment a few of these mice (and all those which received similar injections of phenylcarbamido-insulin) died during the immunization. A similar control group of mice received injections of a solution containing 0.9% NaCl and 0.2% phenol. At various periods after the last injection (4, 9 and 17 days), these two groups of mice and another group of untreated mice were tested for their response to insulin. The technique employed was that of Trevan & Boock [1926]: the mice received subcutaneous injections of 0.015 (or 0.030) unit per 20 g. and were kept at 38° during the assay. No significant difference between the three groups was observed, and the conclusion was reached that injections of large amounts of carbobenzyloxy-insulin had not led to any detectable change in the response of these mice to subsequent insulin treatment.

The serum of the mice "immunized" against carbobenzyloxy-insulin was tested, in complement-fixation and precipitin tests, against carbobenzyloxy-insulin and carbobenzyloxy-gelatin. The results were all negative.

## DISCUSSION

The reaction between benzyl chloroformate and proteins appears to take place mainly if not entirely between the reagent and the free amino-groups of the protein. The reaction is accompanied by the total disappearance of free amino-N, as determined by "formol" titration, and the blocking of the amino-groups is apparently complete. This view is supported by the results of the immunological investigations, for the reaction between carbobenzyloxy-proteins and their antisera is completely inhibited by carbobenzyloxy-amino-acids. To a lesser degree, phenylcarbamido-amino-acids inhibit this precipitin reaction, and this can be attributed to the structural relationship between the carbobenzyloxy- and phenylcarbamido-amino-acid groups. Cross-reactions of this type are quite frequent in serological inhibition tests [cf. Landsteiner, 1936, pp. 122-32].



The results of the immunological experiments described here provide further evidence for the view that substitution in the free amino-groups of antigenic proteins leads to complete, or almost complete, loss of the original specificity and to the acquirement of a new specificity which is characteristic for the introduced grouping. It seems possible that the introduction of any new

group will cause this radical change in the immunological properties of a protein if the new group is sufficiently prominent [cf. Landsteiner, 1936]. The relatively small size of the methylene group might account for the retention of species specificity in formaldehyde-treated proteins; on the other hand, this retention might be due to incompleteness of the reaction between formaldehyde and the free amino-groups, for there is not universal agreement as to the precise nature of this reaction [cf. review by Jordan Lloyd & Shore, 1938].

The action of benzyl chloroformate on insulin also appears to involve the free amino-groups of the protein. The reaction is accompanied by practically complete loss of the hypoglycaemic power of the hormone, similar to, but possibly more complete than, that effected by phenyl isocyanate [Hopkins & Wormall, 1934, 2]. The minimum amount of benzyl chloroformate needed for complete inactivation is about twice the amount required by theory to react with all the free amino-groups. The fact that the "theoretical" amount of the reagent does not effect complete inactivation might be due to action of the reagent on groups other than amino-groups, or, more probably, to loss of the acid chloride by the action of water. The inactivation of insulin is very rapid and takes place at pH 8-8.5 and at ordinary temperatures. The results offer strong support for the view that the free amino-groups of insulin are of significance in connexion with its physiological activity [Hopkins & Wormall, 1934, 2; Jensen & Evans, 1934; 1935].

The attempts to "immunize" mice with carbobenzyloxy-insulin were made in order to determine (a) whether the injection of large amounts of this insulin derivative would influence the response of the animals to ordinary insulin, and (b) whether carbobenzyloxy-insulin would behave as a full antigen. Immunological investigations on insulin are complicated by the hypoglycaemic action of this substance, but this difficulty is overcome by the use of carbobenzyloxy-insulin, which, by analogy with other proteins, should be antigenic if insulin itself has this function. Any antibodies to the inactivated insulin might be expected to show some capacity to react with unchanged insulin, since antisera to carbobenzyloxy-globulin usually give slight precipitation with the untreated globulin. In the experiments described here there was no indication of antibody formation of this type. The sera of the injected mice contained no detectable antibodies to the carbobenzyloxy-derivatives of insulin or gelatin, and furthermore, these animals showed no abnormality when injected with amounts of insulin which are usually sufficient to produce convulsions. It is not easy to forecast exactly what will happen in an animal if an anti-insulin is produced. Small amounts of the antibody could presumably be "neutralized" by insulin with the formation of a complex which might possess some hypoglycaemic activity, but excess of the antibody might be expected to render the animal diabetic. If the animal survives the immunization, perhaps the only evidence of the formation of an anti-insulin might be a change in the response of the animal to insulin injection. Further information on this point might throw some light on problems of insulin-hypersensitivity and insulin-resistance in man.

The antigenic properties of hormones have been studied by numerous authors during recent years, largely stimulated by the observations of Collip and his colleagues. As far as insulin is concerned, the available evidence is rather conflicting. The previously mentioned work of Lewis [1937] suggests that sensitization to insulin can be produced, and Barral [1935] claims to have produced an antigenic and non-hypoglycaemic "aninsulin" by the action of formaldehyde on insulin for 40 days at 60°. Spaccarelli [1936], on the other hand, found that the uteri of insulin-treated virgin guinea-pigs do not react with

insulin, although the uteri of animals injected with a mixture of serum and insulin react to insulin alone. Interesting observations on the antigenicity of insulin have recently been made by Clutton *et al.* [1938]. These workers immunized rabbits against *O*- $\beta$ -glucosido-*N*-carbobenzyloxytyrosyl insulin and found that although the antisera produced to the insulin derivative failed to precipitate the homologous antigen, they gave precipitates with *O*- $\beta$ -glucosido-*N*-carbobenzyloxytyrosyl horse serum globulin. Further, the antisera to the globulin derivative gave precipitates with the insulin compound. The fact that the insulin derivative inhibited the cross reaction of its antiserum with the globulin derivative indicated that, despite the failure of the insulin derivative to precipitate its antiserum, a definite antigen-antibody reaction does occur. These authors suggest that the physical properties of the antigen may be such that an insoluble antigen-antibody complex cannot be formed. They conclude that coupling with glucosidotyrosine converts insulin into a full antigen. The question of the antigenicity of insulin itself is, however, still undecided, and, as far as observations on individuals hypersensitive to insulin are concerned, it might be fair to say that they do not as yet offer conclusive evidence of the presence in the blood of true antibodies to pure insulin.

#### SUMMARY

1. Benzyl chloroformate, the reagent used by Bergmann for the preparation of carbobenzyloxy-amino-acids, readily acts on serum globulin, gelatin and other proteins, at ordinary temperatures and at pH 7.5-8.5. The reaction appears to be concerned mainly with the free amino-groups of the proteins.

2. The injection of carbobenzyloxy-serum globulin into rabbits leads to the production of antibodies which react with the carbobenzyloxy-derivatives of heterologous proteins. The original species-specificity of the serum globulin is almost completely destroyed.

3. The reaction between carbobenzyloxy-proteins and their antisera is completely inhibited by carbobenzyloxy-amino-acids. The immunologically characteristic groups of such proteins are, therefore, the carbobenzyloxy-amino-groups ( $-\text{NH}\cdot\text{CO}\cdot\text{O}\cdot\text{CH}_2\cdot\text{C}_6\text{H}_5$ ).

4. Benzyl chloroformate rapidly destroys the hypoglycaemic activity of insulin. The amount of the acid chloride needed to effect complete inactivation is about twice the amount required by theory to react with all the free amino-groups of insulin, although smaller amounts produce appreciable inactivation. These observations support the view that the free amino-groups of insulin are important for its hypoglycaemic activity.

5. Mice injected with relatively large amounts of carbobenzyloxy-insulin behaved exactly like the control animals when injected with a "convulsive" dose of insulin. These "immunized" mice showed no significant hypo- or hypersensitivity to insulin, and their sera contained no detectable antibodies to carbobenzyloxy-insulin or carbobenzyloxy-gelatin.

The authors are indebted to the Medical Research Council for a personal grant to one of them (W. E. G.) and for a grant which has, in part, covered the expenses of these investigations. Thanks are also tendered to Messrs Boots Pure Drug Co., Ltd., for a generous supply of insulin.

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## CXII. A METHOD FOR THE ESTIMATION OF "TRUE" SUGAR IN 0.05 ml. OF BLOOD

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A METHOD which gives most nearly the true glucose values for blood should show the following characteristics:

(i) Glutathione and other non-sugar reducing substances are excluded either by the protein precipitants [Somogyi, 1929; 1930] or by using unhaemolysed blood [Herbert & Bourne, 1929; 1930].

(ii) The sugar values obtained are identical with the amounts of sugar fermentable by yeast, and the entire blood sugar so determined is fermentable.

(iii) Good results are obtained when pure glucose is estimated in solution and after addition to blood.

A titration method meeting these requirements was recently described [King *et al.* 1937] for 0.2 ml. of blood. A modification of this method is here described in which the blood sample is 0.05 ml. The protein precipitants used are sodium tungstate and copper sulphate [Somogyi, 1931] and the filtrate is treated with a modified Harding & Downs [1933] copper reagent. The cuprous oxide formed is estimated by the colour produced with a phosphomolybdic acid solution [Folin & Wu, 1929].

With this method, glucose was estimated accurately in pure solutions and after addition to blood. Determinations were made using pure glucose solutions, normal bloods, diabetic bloods and mixtures of these bloods with glucose solutions. The results obtained were identical with those found using the previously described method [King *et al.* 1937].

### EXPERIMENTAL

#### *Technique of the analyses*

(a) *Pure glucose solutions.* The glucose solution (1 ml.) was mixed with 1 ml. of the copper reagent in a wide test tube ( $\frac{3}{4}$  in. diameter). The tube, stoppered with cotton-wool, was placed in a boiling water bath for exactly 10 min. After immediate cooling, the mixture was treated with the phosphomolybdic acid (3 ml.). The colours were read within 15 min. in a Duboscq type colorimeter fitted with an Osram sodium light.

Glucose solutions of concentrations from 0 to 75  $\mu\text{g./ml.}$  (equivalent to from 0 to 300 mg./100 ml. in blood at a 1/40 dilution) were treated as above.

The colorimetric readings are shown in Table I.

Glucose standards in benzoic acid gave identical readings.

(b) *Application to blood.* 1 ml. of blood filtrate (1/40 dilution) was used exactly as the glucose solutions above. The method is as follows. 0.05 ml. of whole blood is pipetted into 1.75 ml. of isotonic sodium sulphate solution, or isotonic sodium sulphate fluoride-thymol solution. 0.1 ml. each of sodium tungstate and of copper sulphate are added and the solutions thoroughly mixed. The mixture is centrifuged. 1 ml. of the filtrate (= 0.025 ml. of blood) is mixed with 1 ml. of the

Table I

$\mu\text{g.}$ of glucose per ml. of "test" solution	Reading (mm.) of 20 $\mu\text{g./ml.}$ "standard" solution	Reading (mm.) of 50 $\mu\text{g./ml.}$ "standard" solution
0	0	—
5	2.5	—
10	5.0	—
15	7.5	—
25	12.5	—
30	15.0	6.0
35	17.5	—
40	—	8.0
50	25.1	—
60	—	12.1
75	—	15.1

mixed copper reagent in a wide test tube ( $\frac{3}{4}$  in. diameter). The tube, stoppered with cotton-wool, is placed in a boiling water bath for exactly 10 min. After immediate cooling, 3 ml. of the phosphomolybdic acid reagent are added. The colour produced is compared with that produced by 1 ml. of a standard glucose solution in benzoic acid, treated in the same way as the blood filtrate.

(*Calculation.* Let  $x$  = concentration of standard in  $\mu\text{g./ml.}$

$$\text{Blood sugar (mg./100 ml.)} = \frac{\text{reading of standard}}{\text{reading of test}} \times \frac{x}{1000} \times \frac{100}{0.025};$$

e.g. with 20  $\mu\text{g./ml.}$  standard

$$\begin{aligned} \text{Blood sugar (mg./100 ml.)} &= \frac{\text{reading of standard}}{\text{reading of test}} \times \frac{20}{1000} \times \frac{100}{0.025} \\ &= \frac{\text{reading of standard}}{\text{reading of test}} \times 80. \end{aligned}$$

Similarly, with 50  $\mu\text{g./ml.}$  standard

$$\text{Blood sugar (mg./100 ml.)} = \frac{\text{reading of standard}}{\text{reading of test}} \times 200.$$

Table II shows a comparison of the titrimetric method [King *et al.* 1937] and the present method. Determinations by both methods were carried out independently.

Table II. *Blood sugar (mg./100 ml.)*

Titrimetric method	Colorimetric method
85	84
85	84
90	89
90	89
72	74
73	74
77	78
103	101
103	101
130	129
130	130
108	109
129	128
164	165
189	186
240	242
216	217
300	302

(c) *Recovery experiments.* These were carried out using both methods, the amount of glucose added in both cases being proportional.

	Quantities (ml.) used	
	Titrimetric method	New colorimetric method
Blood	0.2	0.05
Sodium sulphate	1.2	1.25
Glucose solution in isotonic sodium sulphate	2.0	0.5
Copper sulphate	0.3	0.1
Sodium tungstate	0.3	0.1

The results obtained are shown in Table III.

Table III. *Blood sugar (mg./100 ml.)*

Added equivalent of 50 mg./100 ml. glucose		Added equivalent of 100 mg./100 ml. glucose		Added equivalent of 200 mg./100 ml. glucose	
Titrimetric method	Colorimetric method	Titrimetric method	Colorimetric method	Titrimetric method	Colorimetric method
127	129	103	101	85	86
178	178	203	202	289	291
63	64	67	66	107	108
114	114	165	167	306	309
105	104	—	—	67	67
153	154	—	—	267	266

### Solutions

(1) *Copper reagent. Solution A.* 13 g.  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  are dissolved in water and the volume made up to 1 l.

*Solution B.* 50.0 g.  $\text{NaHCO}_3$  are dissolved by shaking in the minimum amount of distilled water. When all the bicarbonate is dissolved, 40.0 g. anhydrous  $\text{Na}_2\text{CO}_3$  are added to the solution and the mixture shaken until all the carbonate has dissolved. 36.8 g. potassium oxalate are dissolved in successive small quantities of warm water and added to the mixture. Finally a solution of 24.0 g. sodium potassium tartrate in the minimum amount of water is added to the mixture. The volume is made up to 1 l. and the mixture well shaken.

The copper reagent used is prepared freshly each day and is a mixture of exactly equal volumes of solutions A and B.

(2) *Phosphomolybdic acid reagent* [Folin, 1930]. 35 g. molybdic acid and 5 g. sodium tungstate are dissolved in 250 ml. of 0.2 *N* NaOH and boiled for 30 min. Water is added to about 350 ml., 125 ml. 89%  $\text{H}_3\text{PO}_4$  (sp. gr. 1.75) are added, and the volume made up to 500 ml.

(3) *Isotonic sodium sulphate containing fluoride and thymol to prevent glycolysis.* 100 mg. NaF and 10 mg. thymol are dissolved in 100 ml. of isotonic  $\text{Na}_2\text{SO}_4$  (3 g.  $\text{Na}_2\text{SO}_4$ , 10 $\text{H}_2\text{O}$ /100 ml. solution).

(4) *Sodium tungstate* (10 g./100 ml.).

(5) *Copper sulphate* (7 g.  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /100 ml.).

(6) *Stock glucose solution.* 0.1 g. pure glucose is dissolved in saturated benzoic acid solution and the volume made up to 100 ml. This is a permanent standard.

(7) *Standard glucose solutions.* These are prepared by diluting 2, 3, 4, 5, etc. ml., to 100 ml. with saturated benzoic acid solution; giving standards of 20, 30, 40, 50, etc.,  $\mu\text{g.}/\text{ml.}$

## SUMMARY

A colorimetric modification (applicable to 0.05 ml. of blood) of a previously described titrimetric copper method for the determination of blood sugar is described.

The method is believed to give true glucose values.

Estimations on normal and diabetic blood by the new method, and also by the previously described method, give results which are in agreement.

The authors wish to express their thanks to Dr E. J. King for his continual advice and encouragement, and also acknowledge previous preliminary experiments by Dr T. F. Nicholson and Dr Y. Seltzer in this laboratory.

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# CXIII. THE PRECIPITATION OF PROTEINS WITH COMPLEX SALTS

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ATTENTION is directed to a group of protein precipitants which are of interest both from a practical and theoretical point of view. The commonly used protein precipitants are subject to certain limitations. Some of them, such as phosphotungstic, hydroferrocyanic and metaphosphoric acids, precipitate proteins in acid solution only, while only few reagents are known, e.g. salts of heavy metals, which effect precipitation in neutral or weakly alkaline solutions. These reagents suffer from the disadvantage that they tend to denature the proteins. Simple salts, such as  $\text{Na}_2\text{SO}_4$ , work only in high concentrations.

It has now been found that complex salts can be used advantageously as protein precipitants, and from the numerous selection of complex salts available a variety of conditions are possible under which the precipitations can be carried out. Apart from the work of Bungenberg de Jong & Saubert [1936], the literature appears to contain no reference to the general use of complex salts as protein precipitants. These authors used complex salts in certain systems which produce what they term "coacervates", but the systems studied were of a more complicated nature than those in our investigations inasmuch as they comprised protein + complex salts + a third component which was usually an inorganic salt. With regard to the mechanism involved in such precipitations, the results of our quantitative experiments support the view that stoichiometric relations exist between protein and precipitant, as found by Chapman *et al.* [1927] for acid dyes, Meyer *et al.* [1937] for chondroitin sulphuric acid, and by Hermann & Perlmann [1937; 1938] for metaphosphoric acid. We have considered both the qualitative and quantitative aspects of the problem.

## QUALITATIVE EXPERIMENTS

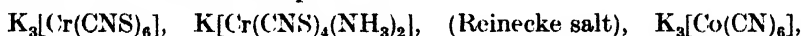
*Methods.* The native protein was dissolved in dilute hydrochloric acid to give a 1% solution having pH 3. A freshly prepared aqueous solution of the complex salt was then added in such amount that the proportion by weight of protein to complex salt was 5 : 1. The appearance or non-appearance of a precipitate was then noted. An analogous procedure was then followed at the other pH values tabulated in Table I. Solutions having a pH value more

Table I

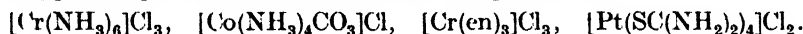
Complex salt	Edestin at pH			Serum albumin at pH			Globin at pH		
	3	4-6	7-9	3	4-6	7-9	3-7.5	8-9	9-11
$[\text{Co}(\text{NH}_3)_6]\text{Cl}_2$	-	+	+	-	+	+	-	+	+
$[\text{Co}(\text{NH}_3)_5(\text{NO}_2)]\text{Cl}$	-	+	-	-	+	-	-	+	-
$\text{K}_2[\text{Cr}(\text{C}_2\text{O}_4)_2]$	+	+	-	+	+	-	+	+	-

+ = precipitate. - = no precipitate.

alkaline than the isoelectric point of the protein were obtained by adding  $N/10$  NaOH until the desired  $pH$  was attained. Precipitation, when it did occur, was instantaneous and the precipitates were flocculent and settled easily. Table I shows that edestin and serum albumin are precipitated in acid solution by the anionic complex salt  $K_3[Cr(C_2O_4)_3]$  (these proteins are normally insoluble within their isoelectric range,  $pH$  4–6); in more alkaline solutions, precipitation occurs with the kationic complex salt  $[Co(NH_3)_6]Cl_3$ . On the other hand, a basic protein, globin, is precipitated in the whole range from  $pH$  3 to 7 by the anionic complex salt  $K_3[Cr(C_2O_4)_3]$ . Within its isoelectric range,  $pH$  8–9, globin is insoluble, while in more alkaline solutions it is precipitated by the kationic complex salt  $[Co(NH_3)_6]Cl_3$ . The complex salt  $[Co(NH_3)_3(NO_2)_3]$ , which is not ionized in aqueous solution, does not cause precipitation of any of the proteins studied, over the whole  $pH$  range. Precipitates of proteins with complex salts redissolve when the  $pH$  of the supernatant solution is altered; thus the precipitates formed by the addition of  $K_3[Cr(C_2O_4)_3]$  to edestin and serum albumin were dissolved when the solutions were adjusted, by the addition of NaOH, to  $pH$  7. The precipitates formed by the proteins with  $[Co(NH_3)_6]Cl_3$  were readily soluble in dilute acid, and the precipitate of globin with  $[Co(NH_3)_6]Cl_3$  was dissolved when the  $pH$  was adjusted to 7. Tests were carried out in the same way with a number of different complex salts. We quote as some typical representatives for the anionic complex salts:



and as typical representatives for kationic complex salts:



It has been found that the precipitation of native proteins can be carried out with all ionized complex salts, provided that anionic complex salts are used at a  $pH$  which is more acid than the isoelectric point of the protein, and kationic complex salts are used at a  $pH$  which is more alkaline than the isoelectric point of the protein. With a proper choice of complex salts precipitation of proteins can, therefore, be effected over a wide  $pH$  range.

### QUANTITATIVE EXPERIMENTS

An analytical study of some precipitates obtained with protein and complex salts was now made. Both types of complex salts, anionic and kationic, were used in this investigation, the precipitations being carried out under the general conditions outlined above.

*Experimental procedure.* The proteins under examination were dissolved in dilute acid or alkali and the solutions then adjusted to a known  $pH$  value. The complex salts which served as precipitants were dissolved and, when necessary, the  $pH$  value of the solution was adjusted to that of the protein solution. In each case the two solutions were mixed and the precipitate which formed was immediately centrifuged off, washed 4–5 times with water until the washings contained no protein or complex salt, washed twice with alcohol, then twice with ether, and finally dried in a vacuum over  $CaCl_2$ . The relative amounts of complex ion and protein in the precipitate were then estimated by analysis. The metallic content of the precipitate was determined and in one case the N contents of the precipitate and the original protein were compared. The experiments were confined to complex salts which contained chromium as central atom, because the method of Smith & Sullivan [1935] for chromium determinations in organic compounds offered a reliable and rapid method for

the estimation of the metal. The nitrogen determinations were carried out using Pregl's micro-Kjeldahl method.

*Exp. 1. Examination of a typical precipitate from a protein and a complex salt.* A crude preparation of insulin<sup>1</sup> (200 mg.) was precipitated in *N*/20 HCl (20 ml.) with a 5% solution of  $K_3[Cr(C_2O_4)_3]$  (1.2 ml.).

Table II

Protein	% N content	% Cr content	% $[Cr(C_2O_4)_3]$ in ppt. calc. from	
			Nitrogen	Chromium
Crude insulin	12.3	—	—	—
Insulin chromic oxalate	10.97	1.72	10.4	10.4

Table II shows that the amount of the complex anion  $[Cr(C_2O_4)_3]$  in the precipitate (as estimated from the Cr content of the precipitate) is identical with the amount calculated from the difference in N content between the original protein and the precipitate. The Cr content of the precipitate may, in this instance, therefore, be taken as a true indication of the amount of complex ion present in the precipitate. A comparison between N and Cr contents could be carried out only in the case of chromic oxalate, because all other complex salts used in these experiments contained nitrogen.

*Exp. 2. (a) Precipitation of protein in acid solution with an anionic complex salt.* In this series both "crude" insulin and crystalline insulin were precipitated with varying amounts of the complex salt  $K_3[Cr(CNS)_6]$ . The precipitations were carried out either in *N*/20 HCl or in 2% acetic acid solution. The conditions were varied with respect to the relative amounts of protein and complex salt, to the concentrations of the protein and the complex salt and to the pH. The reactions were carried out in small centrifuge tubes, the final volume of the mixture always being adjusted to 10 ml.

Table III. *Precipitation of insulin with  $K_3[Cr(CNS)_6]$  in acid solution*

Protein	Wt. of protein mg.	Wt. of $K_3[Cr(CNS)_6]$ mg.	% Cr in ppt.	% Cr in ppt. (average)	% $[Cr(CNS)_6]$ in ppt. calc. from % Cr
(a) Precipitation in <i>N</i> /20 HCl					
Crude insulin	200	60	1.62	1.62	12.45
"	200	60	1.74		
"	200	60	1.64		
"	200	60	1.55		
"	200	60	1.57		
"	200	80	1.69	1.69	13.0
"	200	90	1.64	1.61	12.4
"	200	90	1.57		
"	200	100	1.62	1.62	12.45
"	200	150	1.61	1.61	12.4
"	150	60	1.60	1.60	12.3
"	250	60	1.62	1.62	12.45
"	300	60	1.54	1.54	11.9
Crystalline insulin	200	60	1.70	1.70	13.1
(b) Precipitation in 2% acetic acid					
Crude insulin	250	75	1.57	1.57	12.1
"	250	115	1.59	1.59	12.2

Table III shows that the amounts of complex ion  $[Cr(CNS)_6]$  combined with the protein (as determined by the chromium content of the precipitates) were constant within the limits of experimental error (*ca.* 10%).

<sup>1</sup> A specimen of insulin which was not fully purified and not biologically standardized.

(b) *Precipitation of a protein with a kationic complex salt.* A series of experiments, analogous to those described above under (a), was carried out using the kationic complex salt  $[\text{Cr}(\text{CO}(\text{NH}_2)_2)_6]\text{Cl}_3$ . Casein was chosen as the protein since it is readily soluble at pH 7, and this pH is sufficiently removed from the isoelectric point of the protein. The casein was dissolved in dilute NaOH to give a 1% solution having pH 7. An aqueous solution of the complex salt was added. The relative amounts of protein and complex salt were varied.

Table IV. *Precipitation of casein with  $[\text{Cr}(\text{CO}(\text{NH}_2)_2)_6]\text{Cl}_3$  at pH 7*

Protein	Wt. of protein mg.	Wt. of $[\text{Cr}(\text{CO}(\text{NH}_2)_2)_6]\text{Cl}_3$ mg.	% Cr in ppt.	% Cr in ppt. (average)	% $[\text{Cr}(\text{urea})_6]$ in ppt.
Casein	200	40	1.05	1.05	8.35
"	200	60	1.07	1.07	8.5
"	200	60	1.07		
"	250	25	0.93		
"	250	25	0.87	0.95	7.55
"	250	25	1.06		
"	250	80	1.00	1.00	7.95
"	250	100	1.06	1.08	8.65
"	250	100	1.11		

Table IV shows that whereas the weight of complex salt added was varied by as much as 400%, the maximum variation in the amount of complex ion in the precipitate was only 12%. It appears from the results shown in Tables III and IV that a given protein, when precipitated with a complex salt, combines with it in stoichiometric proportions, which are independent of concentration and relative amounts of protein and complex salt present.

*Exp. 3. Determination of equivalent of complex ion combined with protein in the precipitate.* (a) *Anionic complex ions.* In the experiments recorded in Table V each of the proteins was precipitated with three different complex salts, two of which had trivalent complex anions, while the third (Reinecke salt) had a monovalent complex anion. The figures in column 3 show the percentage of complex ion in the precipitates. It will be seen that for a given protein these values vary considerably for different precipitants. When, however, these figures are recalculated with reference to the equivalent weights of the complex ions and expressed for 1 g. protein (shown in column 4) it will be seen that the

Table V. *Precipitations with anionic complex salts in acid solution*

1 Protein	2 Complex ion*	3 % Complex ion in ppt.	4 M.-equiv. of complex ion combined with 1 g. protein	5 Average
Crude insulin	$[\text{Cr}(\text{CNS})_6]'''$	12.4	1.06	—
"	$[\text{Cr}(\text{C}_2\text{O}_4)_3]'''$	10.6	1.13	1.04
"	$[\text{Cr}(\text{CNS})_4(\text{NH}_3)_2]'$	22.6	0.92	—
Crystalline insulin	$[\text{Cr}(\text{CNS})_6]'''$	13.1	1.12	1.12
Egg albumin	$[\text{Cr}(\text{CNS})_6]'''$	9.4	0.78	—
"	$[\text{Cr}(\text{C}_2\text{O}_4)_3]'''$	7.0	0.715	0.75
"	$[\text{Cr}(\text{CNS})_4(\text{NH}_3)_2]'$	19.4	0.76	—
Clupein	$[\text{Cr}(\text{CNS})_6]'''$	34.1	3.90	—
"	$[\text{Cr}(\text{C}_2\text{O}_4)_3]'''$	30.4	4.15	4.1
"	$[\text{Cr}(\text{CNS})_4(\text{NH}_3)_2]'$	58.0	4.35	—

\* The salts used were the potassium salts.



equivalents of the complex ions combined with a given protein are essentially the same (within the limits of experimental error), and moreover are independent of the nature and the valency of the complex ion used. It is also evident from these figures that the trivalent complexes combine with the proteins as "tribasic acids", while the monovalent complex combines as a "monobasic acid".

(b) *Kationic complex ions.* In the experiments recorded in Table VI various proteins were precipitated in approx. neutral solution with kationic complex salts. The two complex salts used have trivalent complex kations, but their

Table VI. *Precipitations with kationic complex salts at pH 7-8*

1 Protein	2 Complex ion*	3 % Complex ion in ppt.	4 Complex combined m.-equiv./g. protein	5 Average
Casein	$[\text{Cr}(\text{urea})_6]^{+++}$	8.30	0.66	0.68
"	$[\text{Cr}(\text{NH}_3)_6]^{+++}$	3.52	0.71	
Amorphous insulin	$[\text{Cr}(\text{urea})_6]^{+++}$	5.3	0.41	0.415
"	$[\text{Cr}(\text{NH}_3)_6]^{+++}$	2.15	0.42	
Crystalline insulin	$[\text{Cr}(\text{urea})_6]^{+++}$	4.8	0.37	0.37
Egg albumin	$[\text{Cr}(\text{NH}_3)_6]^{+++}$	1.95	0.39	0.39

\* The salts used were the chlorides.

molecular weights (412 and 154 respectively) are far apart. Thus the combining weights of these complex ions differ considerably, and this difference is significant, inasmuch as it excludes any accidental agreement of results. The values found for the two complex ions combined with a given protein are in fairly good agreement. Thus casein combined with 0.66 and 0.71 m.-equiv./g. of protein in the case of  $[\text{Cr}(\text{urea})_6]\text{Cl}_3$  and  $[\text{Cr}(\text{NH}_3)_6]\text{Cl}_3$  respectively. The agreement between the values of the two complex ions is even better in the case of amorphous insulin (0.41 and 0.42 m.-equiv./g. of protein respectively). The corresponding figure for crystalline insulin is slightly lower (0.37). These results show that a protein combines on the alkaline side of its isoelectric point with a definite equivalent of complex kations, independently of the nature of the complex salt used.

The precipitation of proteins with complex salts appears, therefore, to take place in such a manner that each protein displays a definite capacity for combining with acid or basic complex ions.

The averaged values of acid- and base-binding capacities shown in column 5 of Tables V and VI are compared in Table VII with values which have been found by other methods.

The values for insulin found by precipitation with complex anions are slightly higher than the values obtained by Harington & Neuberger [1936] by means of electrometric titration. The figure (0.75), calculated from our experiments for egg albumin, is higher than the value (0.61) which Meyer *et al.* [1937] found from precipitation experiments with chondroitin sulphuric acid, but is in good agreement with the figure (0.78) obtained by Perlmann & Herrmann [1938] from precipitation experiments with metaphosphoric acid. There is good agreement between our value for clupein and that of Felix & Mager [1937] calculated from the combination of clupein with various acids.

Our values for the base-combining capacities of proteins are of the same order as those found by other methods in the case of insulin and casein. Our value for egg albumin, on the other hand, is much smaller than the value of the "maximal base-combining capacity" given by Cohn *et al.* [1925].

Table VII. *Acid- and base-combining capacities of some proteins determined by different methods*

Protein	Acid-combining capacities m.-equiv./g. protein		Method	Author
	Complex ppt.	Other methods		
Amorphous insulin	1.04	—	—	—
Crystalline insulin	1.12	1.01	Electrometric titration	Harington & Neuberger
Egg albumin	0.75	0.61	Precipitated with chondroitin sulphuric acid	Meyer <i>et al.</i>
	—	0.78	Precipitated with $\text{HPO}_3$	Perlmann & Hermann
Clupein	4.1	4.1	Combination with acids	Felix & Mager
Protein	Base-combining capacities m.-equiv./g. protein		Method	Author
	Complex ppt.	Other methods		
Amorphous insulin	0.41	0.45	Electrometric titration	Harington & Neuberger
Crystalline insulin	0.37	—	—	—
Casein	0.68	0.55	Ca-salts	Van Slyke [1915]
Egg albumin	0.39	0.8	Maximal base-combining capacity, electrometric titration	Cohn <i>et al.</i>

### DISCUSSION

Attention is drawn to the fact that all ionized complex salts possess the capacity of precipitating soluble proteins from their solutions. Salts which possess a complex anion precipitate proteins only when the latter are in kationic state, i.e. at a  $pH$  more acid than the isoelectric points of the proteins; salts which possess complex cations precipitate proteins only when the protein is present in its anionic state, i.e. at a  $pH$  more alkaline than the isoelectric point of the protein. This rule applies to all complex salts and all native proteins which have been examined. The precipitation appears to be, therefore, the effect of a salt-like combination of the complex ion with the protein carrying the opposite charge.

We can conclude that the complex ions remain intact in the precipitates from the fact that precipitation is instantaneous and that the colour of the complex ion is retained in the precipitate: e.g. protein precipitates with Reinecke salt are red, with  $[\text{Cr}(\text{urea})_6]\text{Cl}_3$  green, with  $[\text{Cr}(\text{NH}_3)_6]\text{Cl}_3$  orange-yellow. The precipitations described here are, therefore, different in character from those which Elöd & Schachowsky [1935] observed when gelatin was heated with certain unstable complex salts.

The results of our analytical determinations indicate that proteins, when precipitated with a complex salt, combine in stoichiometric proportions with the complex kation or anion respectively. This observation finds a parallel in the combination of certain amino-acids with complex salts [Bergmann, 1935], e.g. *l*-proline rhodanilate,  $[\text{Cr}(\text{CNS})_4(\text{C}_6\text{H}_5\text{NH}_2)_2] \cdot (\text{C}_5\text{H}_{10}\text{O}_2\text{N})$ , in which case the monovalent complex anion is combined with the monovalent amino-acid, the proline reacting in its kationic form.

We can conclude from our figures that proteins combine in acid solution with the complex anion with their "maximal acid-combining capacity", i.e. that all basic groups of the protein are able to react with the precipitant. This is shown in the case of insulin by the agreement of our figure with the highest value of acid-combining capacity found by electrometric titration; it is also in agreement

with Chapman's observation that acid dyes combine with proteins at pH 2-2.5 in constant proportions and in amounts which are to be expected when all basic groups are ionized. Our figures for the base-combining capacities of proteins indicate, on the other hand, that the proteins, when precipitated with kationic complex salts at or near pH 7, do not combine with their "maximal base-combining capacity". A possible explanation is that the phenolic hydroxyl groups are not ionized and therefore do not react.

That precipitation of proteins with complex salts is not accompanied by coagulation or denaturation of the protein is shown by the ease with which the precipitates are redissolved on appropriate change of pH, and by the fact that the precipitations are often reversed by certain neutral salts, such as acetates, phosphates and ammonium salts.

The degree of precipitation of a given protein varies considerably with different complex salts, but we have not detected any considerable differences in the degree of precipitation of different proteins by one and the same complex salt.

#### SUMMARY

1. It has been shown that ionized complex salts precipitate proteins from their aqueous solutions.
2. Anionic complex salts precipitate proteins at a pH more acid than the isoelectric point of the protein, whereas kationic complex salts precipitate at a pH more alkaline than the isoelectric point.
3. These precipitations are not accompanied by denaturation or coagulation of the protein.
4. Quantitative estimations of the N and metal contents of such precipitates have shown that the proteins combine with a definite equivalent of complex ion, independently of the relative proportions and concentrations of the reagents, i.e. in stoichiometric proportions.
5. The amounts of complex ions combined in the precipitates with various proteins represent, with some reservations, the acid- and base-combining capacities of the proteins; they were in several cases found to be in reasonable agreement with values calculated from other determinations.
6. It appears that proteins, when precipitated in acid solution with complex anions, combine with their maximal acid-combining capacity, whereas proteins, when precipitated with complex kations in approximately neutral solution, combine with only a fraction of their maximal base-combining capacity.

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# CXIV. THE ISOLATION OF 17-KETOSTEROIDS FROM THE URINE OF NORMAL WOMEN

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IN a recent paper [Callow, 1939] a chromatographic method of separating the ketones in the neutral fraction of an extract of men's urine was described. By this method androsterone, aetiocholan-3( $\alpha$ )-ol-17-one and *trans*dehydroandrosterone could readily be isolated in greater yields than had previously been possible, and actually in amounts probably not very different from those originally present in the crude extract. The present paper describes the application of the same process to an extract of normal women's urine with the object of making a roughly quantitative comparison.

## EXPERIMENTAL

*Collection and extraction of urine.* Combined extracts from a large number of experimental extractions of bulked normal women's urine, and from the urines of a few normal patients, were used. Most of the bulk collections were made in the Nurses' Home of the Middlesex Hospital. Only extracts of fresh urine, with pH from 5.5 to 7 before hydrolysis, were included. The urine was either hydrolysed by heating with HCl and then extracted with benzene, or a combined hydrolysis and extraction was carried out, as in the method of Dingemans *et al.* [1937; cf. Callow *et al.* 1939]. The extracts were separated into neutral, acidic and phenolic fractions. After colorimetric and capon assays had been carried out, alcoholic solutions of the neutral fractions were combined and the alcohol was removed. The residue from 114 l. of urine was taken up in 200 ml. of methanol, left overnight at 0° and filtered. Evaporation of the methanol gave 3.6 g. of residue. Treatment with the Girard-Sandulesco reagent P gave 1.14 g. of ketonic fraction, i.e. 10 mg./l. of original urine, and 0.91 g. of non-ketonic fraction. Colorimetry indicated 55% of 17-ketosteroids in the ketonic fraction [cf. Callow *et al.* 1938] and a capon assay, for which we are indebted to Mr C. W. Emmens, an androgenic activity of 1.45 I.U./mg., or 14.5 I.U./l. of original urine. The non-ketonic fraction contained 0.25 I.U./l. Half of this brown, gummy ketonic fraction, equivalent to 57 l. of original urine, was taken up in 25 ml. of carbon tetrachloride and put through a 27 × 1.5 cm. column of alumina as previously described. After development with about 800 ml. of carbon tetrachloride, it showed the following coloured bands, reading from top to bottom—brown, red, white, faint pink, faint yellow, white, yellow. Successive portions of 650–800 ml. of the eluate were evaporated, yielding fractions I, II and III, wt. 26, 16 and 6.5 mg. respectively. Fraction I was a gum (72% ketone); Fraction II crystallized partially on standing, but the crystalline material has not yet been identified; Fraction III included the lowest yellow band. Evaporation of a further portion of about 700 ml. of carbon tetrachloride eluate gave only a trace of gummy residue, which was discarded. A total of 2900 ml. of carbon tetrachloride had been used at this stage. The column was then eluted with a total of 1900 ml. of carbon tetrachloride containing 0.1% of alcohol. Evaporation of successive

portions of eluate gave 7 fractions (IV–X), details of which are given in Table I. As with the extract of men's urine, this treatment caused a sharply defined yellow band to appear and to be washed rapidly down the column (Fraction V). Fractions VII, VIII, IX and X were crystalline.

Table I

Fraction	Vol. of eluate ml.	Solvent	Wt. of fraction mg.	M.P. (crude fractions) ° C.	$[\alpha]_D^{25}(\text{EtOH})$	$[\alpha]_{440}^{25}(\text{EtOH})$	Remarks
—	230	0.1% EtOH in $\text{CCl}_4$	Trace	—	—	—	Discarded
IV	200	"	10	—	—	—	Gum
V	90	"	16	—	—	—	Yellow band
VI	220	"	29	—	+ 45.7°	+ 58.7°	Gum: 8 mg <i>transdehydroandrosterone</i> isolated
VII	200	"	11	126–161	+ 85°	+ 110°	Chiefly <i>androsterone</i>
VIII	280	"	35	166–178	+ 96°	+ 120°	<i>Androsterone</i>
IX	300	"	24	156–180	+ 92°	+ 108°	"
X	380	"	5.7	140–178	—	—	"
XI	300	0.2% EtOH in $\text{CCl}_4$	Trace	—	—	—	Gum
XII	300	"	29	—	+ 80°	+ 100°	
XIII	250	"	24.5	125–144 Tr. 140	+ 101°	+ 123°	Actiocholan-3( $\alpha$ )-ol-17-one
XIV	180	"	15	130–150 Tr. 135	+ 100°	+ 120°	"
XV	600	"	36	110–146	+ 90°	+ 110°	Actiocholan-3( $\alpha$ )-ol-17-one + red colouring matter
XVI	430	"	5.7	—	—	—	Gum

Elution was continued with carbon tetrachloride containing 0.2% of alcohol. The first 1630 ml. of eluate were divided into five fractions (XI–XV, Table I), the last of which included the red colouring matter. Further elution (XVI and subsequent fractions) gave small quantities of gummy material which were not examined further.

*Isolation of steroids from crude fractions.* (a) *transDehydroandrosterone*. Fractions IV and V were not further examined. From the behaviour of the extract of men's urine it was suspected that *transdehydroandrosterone* would be found in Fraction VI, which was a yellow gum, weighing 29 mg. Its specific rotation was comparatively low, and colorimetry indicated 67% of 17-ketosteroids. The fraction was treated with 150 mg. of digitonin in 14 ml. of 60% aqueous alcohol. The precipitate which formed overnight was collected and washed with a little ether. It weighed 49 mg. It was then decomposed with pyridine, the digitonin precipitated by the addition of ether, and the solution filtered, washed with dilute HCl and water, dried and the ether removed. The crystalline residue weighed 8.5 mg., and had m.p. 127–140° and  $[\alpha]_D^{25} + 18^\circ$  (EtOH). The identity of the compound with *transdehydroandrosterone* was established by preparation of the benzoate. This was very sparingly soluble in methanol; recrystallized from ethyl acetate, it had m.p. 243–252°, and a mixture with an authentic specimen of *transdehydroandrosterone benzoate* had m.p. 250–254.5° (soft at 243.5°).

The melting point and specific rotation suggested that Fraction VII might be a mixture of *androsterone* and *transdehydroandrosterone*. An approximate absorption spectrum of the colour produced with Zimmermann's reaction showed that it was about 90% 17-ketosteroid. It was taken up in 1.5 ml. of 50% aqueous alcohol and treated with a hot solution of 80 mg. of digitonin in 8.5 ml. of 50% alcohol. After standing overnight the precipitate was collected and weighed. Yield: 7.5 mg. This was decomposed in the usual way and the product benzoylated, when the characteristic benzoate of *transdehydroandrosterone* was obtained. The total yield of *transdehydroandrosterone* from Fractions VI and VII was about 10 mg.

(b) *Androsterone*. The filtrate from the above digitonin precipitate was extracted with ether, the extract washed with water, dried and the ether removed. The slightly gummy crystalline residue weighed 12 mg. Recrystallized from methanol it melted at 178–181.5°, after subliming on to the cover slip. It was combined with later androsterone fractions for further recrystallization. The specific rotations and melting points of the crude Fractions VIII and IX suggested that they were androsterone. An approximate absorption spectrum of the colour reaction was that of a 17-ketosteroid. Fraction VIII recrystallized from methanol gave plates, m.p. 181–183°, after subliming to needles, and, mixed with an authentic specimen of androsterone, melted at 179–183°. The identity of the compound as androsterone was confirmed by preparation of the oxime. This, recrystallized from aqueous alcohol, had m.p. 208–211°, which was unchanged by admixture with an authentic specimen. Fraction IX recrystallized from methanol gave 10 mg. of plates m.p. 182–184° after subliming to needles. Mixed with an authentic specimen of androsterone, the m.p. was 183–185°. The total amount of androsterone isolated in a moderately pure state was 76 mg. It may be noted that this quantity accounts for 90 % of the biological activity of the crude ketonic fraction as measured by the comb-growth method.

(c) *Aetiocholan-3( $\alpha$ )-ol-17-one*. The crystalline Fractions XIII, XIV and XV were separately treated with charcoal in methanol solution. Addition of water to the filtrates gave material crystallizing in long needles which partially melted and changed to a platy crystalline form at 140–143° and finally melted at about 150°. This behaviour suggested that the material in all cases was aetiocholan-3( $\alpha$ )-ol-17-one, which has a transition point at 140–142°, but a sharp m.p. could not be obtained. The fractions were therefore combined and converted into the benzoate. This had m.p. 161.5–163.5° (from methanol) and the m.p. was unchanged by admixture with an authentic specimen of aetiocholan-3( $\alpha$ )-ol-17-one benzoate. About 76 mg. of aetiocholan-3( $\alpha$ )-ol-17-one were isolated in a moderately pure state.

(d) *Unidentified material*. In addition to the small amount of crystalline material separating from Fraction II already mentioned, there is an indication of an unidentified material in the non-crystalline Fraction XII. The position in the series, the specific rotation (see Table I) and the 17-ketosteroid content (72 %) suggest that it might be a mixture of aetiocholan-3( $\alpha$ )-ol-17-one with a non-ketonic impurity, but the presence of other compounds cannot be excluded. A crystalline benzoate, m.p. 80–140°, was prepared, but the amount was not sufficient for purification. Further investigation of these materials will require larger amounts of urine extract.

*Note.* All melting points recorded were observed in Kofler's micro-melting point apparatus. The optical rotations were measured in a 4 dm. tube.

## DISCUSSION

The origin of the 17-ketosteroid compounds in women's urine is as yet a matter only for surmise. Androsterone and aetiocholan-3( $\alpha$ )-ol-17-one were isolated in abnormally high yield from the urine of a man receiving testosterone [Callow, 1939]. This observation indicates that testosterone is transformed by the body into androsterone and aetiocholan-3( $\alpha$ )-ol-17-one. The further observations that these two compounds were major constituents of the neutral ketonic fraction of extracts of normal men's urine was consistent with the assumption that they were derived from endogenous testosterone, assumed to be secreted by the testis. The observations now reported show that normal women's urine yields 1.3 mg./l. of androsterone and 1.3 mg./l. of aetiocholan-3( $\alpha$ )-ol-17-one, as

compared with the yields of 1.6 mg./l. and 1.4 mg./l., respectively, now obtained from normal men's urine by the same method. The excretion of these two compounds is, therefore, not an index of the secretory activity of the testis. The simple assumption that these compounds are excretory transformation products characteristic of testosterone only, would lead to the conclusion that testosterone was secreted by the normal woman in amounts comparable with those secreted by the normal man.

There are, however, no grounds for the assumption that these compounds cannot also be derived from other steroids in the body, whether of gonadal or of adrenal cortical origin. Although ovaries may secrete material with androgenic activity [Hill & Gardner, 1936; Parkes, 1937; Hill & Strong, 1938; Deanesly, 1938], the nature of the compounds produced is unknown. The idea that normal males and females both secrete the same highly active androgen in comparable amounts is contrary to current physiological theories, although Korenchevsky [1939] considers it reasonable to suppose that male hormones present in the female should be of the same nature as the other natural male hormones. The next step we propose to take in the chemical investigation is to examine the nature of the compounds excreted by eunuchs and ovariectomized women. Presumably this will give a clue to the contribution of the adrenal cortex to the urinary 17-ketosteroids. It may be that the latter will include the compounds now regarded as derived from testosterone. Up to the present the evidence points to *transdehydroandrosterone* being a characteristic transformation product from the adrenal cortex in tumour cases [Callow, 1936; Crooke & Callow, 1939], whilst Butler & Marrian [1938, 1, 2] have isolated *aetiocholan-3( $\alpha$ )-ol-17-one* and *androstan-3( $\beta$ )-ol-17-one* from the urine of a case of adrenal hyperplasia. It may be noted that the amounts of *transdehydroandrosterone* now isolated from urine of normal women, although higher than those obtained previously [Callow & Callow, 1938], are still small and comparable with those from normal men's urine.

#### SUMMARY

1. *Aetiocholan-3( $\alpha$ )-ol-17-one* (1.3 mg./l.), *androsterone* (1.3 mg./l.) and *transdehydroandrosterone* (0.2 mg./l.) have been isolated from extracts of the urine of normal women. These yields are comparable with those of the same compounds from normal men's urine.

2. *Androsterone* is the principal androgenic compound in extracts from the urine of either sex.

3. Although urinary *aetiocholan-3( $\alpha$ )-ol-17-one* and *androsterone* are known to be derived from testosterone when this has been given by injection, it does not follow that their presence in urine indicates the secretion of testosterone by women.

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# CXV. GROWTH AND REPRODUCTION ON A LOW FAT DIET

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THE low fat diet employed by the Burrs [Burr & Burr, 1929; 1930; Burr *et al.* 1932] in their work on the essential fatty acid has served as a model in the search for new fat-soluble dietary essentials. The diets used in such investigations have, without sufficient evidence, been generally referred to as "fat-free", "rigorously fat-free" etc. Failures to disclose the existence of new factors of a lipid nature are qualified, therefore, by the absence of quantitative data on the fat contents of these diets. In addition, there exist in the literature suggestions, both implied in the results reported and specifically stated, that fat-soluble factors other than vitamins A, D and E, and the essential fatty acid, are required by the rat [Burr & Burr, 1929; Mason, 1929; Coward *et al.* 1929; Guha, 1931; Mapson, 1932; Blumberg, 1935; Marchesi, 1935; Olcott & Mattill, 1937; Martin, 1939].

When analysis disclosed that several components of so-called "fat-free" diets contained appreciable amounts of lipid, the preparation of a diet lower in lipids than any previously fed was successfully attempted. The analytical findings, the preparation of the diet and the results of the subsequent feeding experiments are reported in this paper.

## *Analysis of dietary components and preparation of a low fat diet*

"Fat-free" diets have generally been composed of a salt mixture, sucrose, casein (ether- or alcohol-extracted or both), ether-extracted yeast and pure or concentrated sources of vitamins A (or carotene), D and E, and the essential fatty acid. Since the degree of purity of the fat-soluble essentials used in these diets is generally apparent, and since methods for purifying them are available, attention was first turned to the lipid contents of extracted yeast and casein.

*Analysis of dietary components.* The Liebermann saponification method for fat determination as described by Leathes & Raper [1925] was found most suitable for the analysis of yeast. By acidifying and extracting the entire saponified mixture, a measure of all light petroleum-soluble substances was obtained.

Table I. *Lipid content of dried yeast before and after ether extraction*

Brand of yeast	Lipid content %		
	Unextracted	Ether-extracted 24 hr.	Ether-extracted 48 hr.
Northwestern Yeast Foam	4.2	3.7	3.7
Tablet Powder			
Anheuser-Busch Strain C	5.7	5.5	5.5
Fleischmann No. 1550	5.8	4.6	4.5

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Three brands of dried yeast, unextracted and extracted for periods of 24 and 48 hr. in the Soxhlet apparatus with C.P. anhydrous ethyl ether, were examined. The average figures (Table I) show differences in the yeasts both with respect to total lipids and the % lipids removed by ether extraction; but in each case it is clear that most of the fatty substances could not be removed by this method. Whether this was due to their inaccessibility to the solvent, or to their existence in insoluble chemical combinations was not determined.

In order to eliminate the possibility of the conversion of sugars or amino-acids into light petroleum-soluble substances during the strong alkali hydrolysis, control experiments were made with sucrose and casein but with negative results. Furthermore, ether-extracted yeast cells stained for fat with Sudan III gave positive results, as evidenced by the appearance of many bright red globules. Thus the assumption that ether-extracted yeast furnishes a "fat-free" source of the vitamin B complex was shown to be erroneous.

Two methods, the Liebermann saponification [Leathes & Raper, 1925] and the Shaw [1920] adaptation of the Reese-Gottlieb procedure, were investigated as to their accuracy in determining the lipid content of casein. Since the Liebermann method gave much lower values owing to the formation of a fat-including coagulum, the method of Shaw was accepted as superior in this case. The results of analyses made on samples of casein subjected to extraction with hot or cold alcohol and ether, or to successive extractions with these solvents, are given in Table II. Ether extraction of crude casein is much less

Table II. *Lipid content of extracted casein*

Casein	Treatment	Lipid content %
Crude	None	1.45
Crude	None*	1.50
E.E.	Ether-extracted*	1.48
CA	Cold alcohol-extracted†	0.24
CA 1	Cold alcohol + 24 hr. warm ether extraction‡	0.17
CA 2	Cold alcohol + 48 hr. warm ether extraction	0.13
CA 3	Cold alcohol + 72 hr. warm ether extraction	0.12
CA 4	Cold alcohol + 24 hr. hot 95 % alcohol extraction	0.025
CA 5	Cold alcohol + 24 hr. warm ether and 24 hr. hot 95 % alcohol extraction	0.02

\* Figures taken from Shaw [1920].

† Acetic acid-washed and continuously extracted in a Lloyd extractor with cold 70 % and cold 95 % alcohol for 24 hr. each.

‡ This and all subsequent extractions carried out in Soxhlets.

efficient than cold 70 and 95 % alcohol extraction, which removes approximately 83 % of the lipids. Furthermore, it is seen that the removal of lipid from this cold alcohol-extracted casein is more readily accomplished by continuous extraction with hot 95 % alcohol than with warm anhydrous ethyl ether. The successive application of these two last-named solvents does not seem to reduce the lipids to a significantly lower level than extraction with hot 95 % alcohol

alone. Indeed, in both of these cases the results of the analysis are in the range of the blank determinations, but since there generally seemed to be one or two droplets of lipid in the weighing vessels no corrections were made. It would appear from Table II that many "fat-free" diets have contained milk fat in addition to yeast fat.

Since no lipid was found in commercial sucrose analysed by the Liebermann method, the sucrose used in "fat-free" diets has probably not been a source of contamination.

Of the known fat-soluble essentials used in these diets, provitamin A and vitamin D have recently been included in pure forms; vitamin E has, with a few exceptions, been added as the crude unsaponifiable matter of wheat germ oil, and the unsaturated fatty acid has been supplied (in long term experiments) by a distillate containing a mixture of esters.

*Preparation of a low fat diet.* (Low fat diet is used throughout the remainder of this paper to refer to a diet whose lipid content is small.) Following unsuccessful attempts to remove lipids completely from yeast, a satisfactory source of the vitamin B complex was extracted in the following manner. 100 g. Northwestern Yeast Powder were added to 1 l. 50 % alcohol and the mixture was rapidly stirred for 2 hr. The mixture was allowed to stand for 1 hr. and the extract was then filtered through No. 52 Whatman filter paper. Most of the alcohol was removed by concentrating the filtrate on the water pump at 40–50° to a volume of about 200 ml. The concentrate was shaken for 3 min. in a separating funnel with an equal volume of c.p. anhydrous ethyl ether. This extraction was repeated. Liebermann analysis showed the fat content of the resulting defatted yeast extract in terms of whole yeast to be 0.01 %.

The extract was assayed for growth, reproduction and lactation on vitamin B complex-deficient rats. The extract from 100 g. of yeast added to 900 g. of diet produced excellent growth in depleted male rats, an average gain of 300 g. over a period of 17 weeks. Female animals that had gained 120 g. in 6.5 weeks and were then mated produced and weaned good litters. The results obtained with the extract at this level were comparable in every respect with those obtained on 10 % whole yeast.

The preparation of a low fat casein is indicated in Table II (Casein CA5). While ether extraction in addition to hot alcohol extraction had no apparent effect on the lipid content, this step was included as an added precaution. The hot 95 % alcohol extraction was carried out in all-glass Soxhlets.

Methyl linoleate was prepared from cottonseed oil by the method of Rollett [1909]. This procedure was facilitated by substituting a 1:1 light petroleum (B.P. 30–60°): carbon tetrachloride mixture for ligroin in recrystallizing the tetrabromostearic acid. The purity of the preparation was attested by the melting-point of the tetrabromide, 114–115°, the figure obtained by Rollett, and the iodine number of the ester, 172.3, the theoretical value being 172.2.

A method for preparing a highly potent concentrate of vitamin E for long-term feeding experiments where the use of the pure vitamin is not yet feasible has been reported [Mackenzie *et al.* 1938]. Although this concentrate had proved potent in single 3 mg. doses in rats reared on a typical lard-containing vitamin E-deficient diet, there was no assurance that the same level would suffice on a low fat diet. It was assayed at a 5 mg. level on female rats maintained on the low fat diet (described below) without vitamin E and found to be effective.

As the remaining components necessary for the completion of the diet, sucrose, cystine (as a supplement to the casein), salts, calciferol and carotene, presented no problems, the following diet was assembled.

Sucrose	...	...	...	...	...	63.85
Casein (extracted with cold 70 % and 95 % alcohol, warm ether and hot 95 % alcohol)	...	...	...	...	...	20.00
Cystine	...	...	...	...	...	0.05
Salt mixture, McCollum 51* + 1.6 % $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$	...	...	...	...	...	6.10
Yeast extract† equivalent to 10 g. yeast						
Carotene, 20 mg. per 900 g. diet						
Calciferol,‡ 0.2 mg. per 900 g. diet						

\*  $\text{CaCO}_3$  1.5, KCl 1.0, NaCl 0.5,  $\text{NaHCO}_3$  0.7, MgO 0.2,  $\text{KH}_2\text{PO}_4$  1.7, Fe citrate 0.5.

† Extract prepared from Northwestern Yeast Foam Tablet Powder.

‡ We are indebted to Dr C. E. Bills of Mead Johnson & Company, Evansville, Indiana, for the calciferol.

#### Supplements

Methyl linoleate, 25 mg. per rat 6 times weekly.

Vitamin E concentrate, 5 mg. per rat twice weekly.

*Mixing the diet and care of animals.* The yeast extract was poured on the casein and evaporated at room temperature before an electric fan. The carotene and calciferol were dissolved in C.P. anhydrous ethyl ether and immediately evaporated on separate portions of the casein-yeast extract mixture. The various components of the diet were then thoroughly mixed. The diet was prepared fresh every 3–5 days and stored in the ice box between feedings.

All-metal individual cages with raised mesh bottoms were used to house the animals. Food was supplied *ad libitum*, the supply being replenished daily, except once each week when the animals were allowed to consume their ration entirely, thus ensuring the complete intake of all components. Supplements were administered in the morning on a small amount of the basal diet and were always consumed by evening.

The maximum lipid content of the basal diet is 0.0078 %, of which 0.0022 is due to carotene and calciferol and 0.0056 to impurities in the casein and yeast extract. Of the two supplements, the methyl linoleate is pure, while the vitamin E concentrate contains at the most 70 % of extraneous matter.

The daily consumption of lipid, based on the average food intake of 10 g. per rat per day, is approximately 0.027 g., of which 0.025 g. is methyl linoleate. Thus the lipid content of the complete diet (basal plus supplements) may be given as 0.27 %. Similarly, the maximum non-vitamin lipid content of the complete diet is 0.0156 %, and of the diet without vitamin E, 0.0056 %.

#### Feeding experiments on the low fat diet

Animals obtained from the laboratory stock colony<sup>1</sup> were placed on the experimental diet when they were about 21 days old and weighed 35 g. The composite growth curves of one group of 10 male and one group of 10 female rats maintained on the diet for a period of 44 weeks are given in Fig. 1. The curves for the low fat and the stock females show an insignificant superiority in weight on the part of the low fat animals. The curve for males on the low fat diet diverges from the stock male curve at 12 weeks and is about 30 g. below at 44 weeks. The significance of this difference is questionable in view of the great individual variations within both groups.

The average time of cessation of growth (defined as a failure to gain more than 5 g. during the ensuing 6 weeks) for the female rats was 22 weeks. Some females maintained a constant weight until the experiment was terminated, while others after remaining at a constant weight for 7–12 weeks gained as much

<sup>1</sup> The McCollum stock diet is composed of wheat (soft) 20.0, maize (yellow) 20.0, rolled oats 20.0, flaxseed oil meal 10.0, casein (crude) 3.5, whole milk powder 25.0,  $\text{CaCO}_3$  0.5, NaCl 1.0, Fe citrate 0.0011,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.0004.

as 15 g. Eight male rats ceased to grow after an average of 32 weeks, while 4 were still growing at 40 weeks. Of the 8 males, 3 subsequently gained 15–35 g. and 5 held a constant weight for their remaining 8 weeks on the experiment. The erratic behaviour of the animals with respect to the time and duration of constant weight maintenance could not be correlated with different batches of diet.

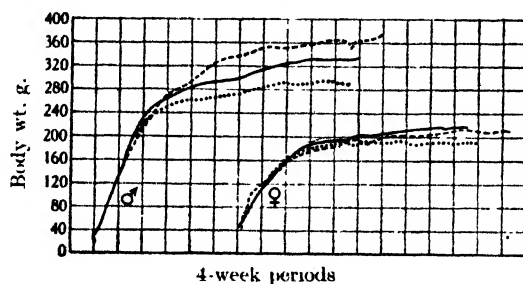


Fig. 1. Average growth curves of male and female rats receiving the low fat diet ———, the low fat vitamin E-deficient diet ..... and the stock diet - - - - -.

The adequacy of the diet for growth was further tested on 4 groups of rats by doubling the intake of all vitamins and cystine, by increasing the vitamin E concentrate four-fold, by giving supplements of fresh calves' brain, and by replacing the low fat diet by the stock diet. With the exception of the experiments in which the vitamin and the cystine intakes were doubled, these changes were made with rats that had maintained constant weights for 6 weeks or longer. The growths of the control and test animals had been similar prior to the changes in diet. The results of these changes, summarized in Table III, were in all cases negative.

Table III. *Growth response as a result of supplementing or replacing the low fat diet for 4- and 8-week periods*

Change in diet	No. of rats	Sex	Av. time of change weeks	Av. time of cessation of growth weeks	Av. wt. at cessation of growth g.	Av. wt. at change g.	Av. wt. increase over wt. at cessation of growth	
							4 weeks	8 weeks
Doubling all vitamins and cystine	3	♂	19	—	—	261	12	—
Controls on low fat diet	3	♂	20	—	—	260	12	—
Doubling all vitamins and cystine	2	♀	21	—	—	162	4	—
Controls on low fat diet	2	♀	20	—	—	182	6	—
Vitamin E concentrate, 40 mg. per week	3	♀	25	18	213	212	2	—
Controls on low fat diet	3	♀	31	25	191	191	4	—
Fresh brain, 1 g. daily	5	♀	28	21	199	196	2	6
Controls on low fat diet	5	♀	28	22	205	204	3	5
Stock diet	2	♂	36	30	279	278	10	14
Controls on low fat diet	4	♂	38	30	304	302	10	10
Stock diet	6	♀	37	30	196	193	1	1
Controls on low fat diet	6	♀	36	27	204	201	1	1

Since brain is an exceptionally rich source of lipids, 4 male and 4 female rats were placed at weaning on the low fat diet with the addition of 1 g. of fresh brain per rat daily and maintained on this regimen for 30 weeks. Their growth did not significantly differ from that of animals on the low fat diet.

The fertility of females on the low fat diet was demonstrated by the delivery of 14 litters by 5 rats mated to stock males. Following the second and third parturitions at 15 and 21 weeks, the behaviour of the mothers and the young was observed. Several days before the termination of pregnancy the mesh bottoms were removed from the cages and the females were placed on a bed of filter paper clippings previously extracted with hot 95 % alcohol. In all cases the females built nests and showed solicitude for their young, as was manifested by retrieving and hovering. Though the young were normal in appearance, many died during the first 3 days. Doubling the vitamin and cystine intakes on the 13th day of the 3rd pregnancy with 3 of the rats did not substantially improve the results. When young were living at 21 days they were, with the exception of those from one litter, poor in weight and appearance. The details of this experiment are summarized in Table IV.

Table IV. *Reproductive behaviour of females grown and maintained on the low fat diet*

Rat no.	First gestation*			Second gestation			Third gestation			
	No. of living young	Av. wt. at birth g.	No. of living young	Av. wt. at birth g.	Fate of young	Av. wt. at 21 days g.	No. of living young	Av. wt. at birth g.	Fate of young	Av. wt. at 21 days g.
C 5	9	4.1	7†	4.7	Dead 1 day	—	5‡	5.8	5 weaned	31.0
C 8	8	5.2	9	5.2	Dead 8 days	—	7‡	5.6	2 weaned	20.5
C 18	7	4.1	8	4.5	Dead 2 days	—	10‡	4.1	Dead 3 days	—
C 24	6	4.7	10	4.6	Dead 1 day	—	11	4.8	1 weaned	19.0
C 30	6	5.5	9	4.9	5 weaned	23.5	—	—	—	—

\* Lactation not observed following 1st gestation.

† In all 2nd and 3rd gestations, litters were reduced to 6 young on day following delivery.

‡ All vitamins and cystine doubled on 13th day of pregnancy.

Results obtained by exchanging the young of the low fat diet mothers with those of stock diet females suggested that milk production on the low fat diet was probably inadequate.

Four male and 4 female second generation animals weaned by females C5, C8 and C30, which had grown up on the low fat diet (Table IV), were kept on this diet for a period of 16 weeks, at which time the average weight for males was 228 g. and for females 165 g. The appearance of these animals was normal.

Following their last gestation, the female rats used for reproduction were continued on the low fat diet for 10–20 weeks. Despite the nutritional strain of pregnancy, and in some cases lactation, they showed no signs of abnormality.

Motile sperms were found in the epididymides of male rats after they had been 44 weeks on the low fat diet.

*Feeding experiment on the low fat vitamin E-deficient diet*

As the possibility existed that the vitamin E concentrate used in the complete low fat diet might, in addition to vitamin E, contain another factor essential for the rat, 10 male and 8 female rats were reared on the low fat diet without vitamin E. The growth of these animals, though somewhat inferior to that of the controls, gave no indication that any factor other than vitamin E was lacking. The average growth curves are given in Fig. 1. The average time when weight became stationary for the 8 females was 16 weeks, and for 8 of the males 23 weeks. Two of the males had not ceased growing at 37 weeks. As was the case with the animals on the complete diet, there was an inexplicable tendency in both males and females to resume growth after maintaining a constant weight for 6-10 weeks. This occurred in about half of the animals and the gains varied between 10 and 20 g. in 9-12 weeks.

After the deficiency of vitamin E in the diet had been checked by resorption gestations on several females, 4 female rats whose weights were stationary were given 20-40 mg. of the vitamin E concentrate weekly. An average gain of 18 g. in 12 weeks resulted, as contrasted with an average gain of 1 g. for their 4 controls. This gain corresponds rather closely to the difference between the growth curves of female rats with and without vitamin E.

The oestrous cycle was followed on 6 rats from the 8th to the 23rd week of the experiment. The average length of the cycle for 5 of the rats was 4.7 days. For the 6th animal the average was 5.8 days. The prolongation of the cycles in this case occurred during post-oestrus.

At the 40th to the 42nd week of the experiment some of the vitamin E-deficient animals showed the first symptoms of the paralysis mentioned by Evans [1932] and later described by Blumberg [1935], Ringsted [1935] and Burr *et al.* [1937]. There was an unmistakable spreading of the hind legs accompanied by a lowering of the posterior abdominal region. This abnormality was observed in 5 of 8 males and 1 of 4 females. Thirteen male and 5 female rats on the low fat diet with added vitamin E concentrate showed no signs of the paralysis at 42-44 weeks.

## DISCUSSION

Inspection of the "fat-free" diets used by others in nutritional investigations, and consideration of the analytical findings reported in this paper, show that in addition to the known fat-soluble dietary essentials such diets have included light petroleum-soluble substances of unknown significance. The diet used in this experiment contained at the most, exclusive of the fat-soluble essentials, 0.0156 % of lipid. Of the fat-soluble essentials, vitamin E alone was added in an impure form, and here the contamination did not exceed 7 mg. of lipid matter per rat per week. The disadvantages resulting from the use of a concentrate instead of the pure vitamin were partially mitigated by running a parallel experiment on the low fat diet without vitamin E, thus reducing the non-vitamin lipid to 0.0056 %. The symptoms of avitaminosis-E would hardly be expected to obscure an additional nutritional deficiency.

The growth of animals maintained on the complete low fat diet for as long as 11 months was good and compared favourably with growth on the stock diet. Modifying the low fat diet, even to the extent of replacing it by the stock diet, did not significantly affect the weights of the animals. The differences between the average growth curves of animals on the complete and vitamin E-deficient diets suggested an uncomplicated vitamin E deficiency. Furthermore, the

response of vitamin E-deficient females to the vitamin E concentrate corresponded to the difference between the growth curves and was of the order of magnitude of the growth response obtained by Evans *et al.* [1938] with pure vitamin E. These results, obtained on the complete and vitamin E-deficient low fat diets, furnish no evidence for the existence of an additional fat-soluble growth factor.

Female rats on the experimental diet were fertile. Since it is not likely that the vitamin E concentrate carried a second factor necessary for reproduction, additional evidence is furnished against the existence of a hitherto unrecognized fat-soluble essential. The subsequent normal behaviour of the females used for reproduction and the fair growth of second generation animals point in the same direction. However, negative results in an experiment of this kind can never be conclusive. While the probability of the existence of an additional fat-soluble growth (and to a lesser degree reproduction) factor has been considerably reduced, it must be borne in mind that experiments in which different basal diets are employed may yield opposite results.

It seems probable that the inferiority of young born to females on the low fat diet was primarily due to inadequate milk production. If this was the case, a subminimal supply of the vitamin B complex can be excluded as the cause, since good lactation was obtained in animals used for assay of the yeast extract. One or more of the fat-soluble vitamins may have been involved, though doubling them did not rectify the condition. Similar results on low fat diets have been reported by Evans *et al.* [1934], and Olcott & Mattill [1937]. The former workers found the addition of 25 % lard or butter fat beneficial. While this suggests that a fat-soluble factor, or fat as such, is necessary for normal lactation, in none of these cases has the complicity of cystine or methionine, shown by Daggs & Tomboulion [1935] and Daggs & Lidfeldt [1938] to be potent lactagogues, been excluded.

The failure of Olcott & Mattill [1937] to observe the paralysis reported by Evans [1932], Blumberg [1935], Ringsted [1935], and Burr *et al.* [1937] was probably due to the comparatively short duration of their experiments. On the low fat vitamin E-deficient diet used in this experiment, the first symptoms of the paralysis were observed in some of the animals at 40–42 weeks. Heretofore the evidence that the paralysis is a result of vitamin E-deficiency has rested on the preventive action of wheat germ oil. The absence of symptoms from control animals receiving a highly potent vitamin E concentrate is an advance in the identification of the active substance.

#### SUMMARY

1. Evidence has been presented indicating the presence of fat in several components of "fat-free" diets.

2. The preparation of a diet lower in lipids than any previously reported has been described. The total lipid content of this diet is approximately 0.27 %. The maximum non-vitamin lipid content of the diet is 0.0156 %, and of the diet without vitamin E concentrate, 0.0056 %. The other fat-soluble essentials were added in pure form.

3. This low fat diet was found satisfactory for good growth and reproduction.

4. The results of feeding experiments on the complete and vitamin E-deficient diets furnish no evidence for the existence of a hitherto unknown fat-soluble factor necessary for growth or reproduction in the rat.

5. The early symptoms of paralysis observed in adult rats on a vitamin E-deficient diet were prevented by a highly potent concentrate of vitamin E.

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# CXVI. THE BIOCHEMISTRY OF SILICIC ACID

## VIII. THE DETERMINATION OF SILICA

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THE method employed for the determination of silica in tissues and body fluids must depend on the amount and nature of the silica present. For tissues such as the lungs of workers in dusty atmospheres, where a great deal of mineral matter may be present, the ordinary gravimetric procedures are applicable. In the case of normal tissues of low silica content a micro-gravimetric method is suitable [Morgan & King, 1932], but a colorimetric method may be preferred because of the absence of the technical difficulties inherent in the micro-gravimetric procedure. The quantities of silica normally encountered in urine and blood are so small as usually to demand a colorimetric method.

The silica of lungs and lymph glands consists largely of particles of mineral dust which have gained access to the tissues through the air passages. Much of it, being derived from refractory silicate rocks, is insoluble in chemical reagents (e.g. quartz, mica). This material must be decomposed before it can be estimated. For this reason it is essential to fuse the tissue, or its ash, with sodium carbonate. The endogenous "silica of constitution" of other tissues is more easily brought into solution. But it is found most convenient to fuse these also with sodium carbonate, when there can be no question that all of the silica in the tissue is being determined. In blood and urine all the silica appears to be in a soluble state. This makes possible a direct determination through a chemical reaction leading to the production of a colour without the necessity of preliminary decomposition with sodium carbonate.

Several alterations and improvements have been made in the methods of silica analysis employed in this investigation since the publication of the first paper of the series [King & Stantial, 1933] on the "micro-determination of silica". These are outlined in the present communication, together with directions for the application of the method to a variety of biological materials, both normal and pathological, and to mineral dusts. The description of a gravimetric procedure is likewise given.

### *Methods*

*Preparation of tissue for analysis.* There is a large degree of variation in the silica content of different parts of some tissues. This is notably the case with lungs, where the inhaled siliceous dust settles in largest amount in the region of the hilum, and becomes concentrated in the root glands. With these tissues it is essential to obtain as representative samples as is possible, so that the analysis may represent the composition of the whole organ; wherever possible the entire organ should be taken [cf. Collins & Dible, 1935]. Tissues like liver and muscle are much more uniform in composition, and need not be sampled so carefully. The analysis is preferably done on dried and powdered tissue rather than on fresh tissue. A block of tissue or the whole organ is trimmed free from adherent material, and is washed first with tap water and then with distilled water. It is

cut in small pieces and placed on metal trays in an oven at  $105^{\circ}$  to dry. After 24 hr. in the oven it will usually have reached constant weight. The hard lumps of dried tissue are pounded to a powder in an iron mortar and again left in the oven overnight. If a power-driven mortar and pestle are available, the reduction of the sample to a fine powder is an easy matter. If it has to be done by hand, the process is facilitated by screening off the finest material several times during the grinding. The powdered dry tissue is finally thoroughly mixed on a sheet of paper, a portion is taken for analysis and the remainder stored in a closely stoppered bottle or in envelopes in a desiccator.

*Gravimetric analysis*—semi-micro-method (for tissues having a high silica content). The lungs of workers who have been exposed to the inhalation of silica dust may contain 0.5–5 % or more silica on the dry weight. An amount of the dry powdered tissue is taken which may be expected to yield 5–50 mg.  $\text{SiO}_2$ . The sample is intimately mixed with 4 times its weight of anhydrous  $\text{Na}_2\text{CO}_3$  in a platinum crucible of 10–20 ml. capacity. The mixture is carefully “smoked off” and heating continued until a clear liquid melt is obtained. The cooled contents of the crucible are dissolved in water on the steam bath. Conc. HCl is carefully added until the mixture is acid to methyl orange, and 3 or 4 ml. extra added. The mixture is evaporated to dryness on the steam bath, and heated for 1 hr. at  $105^{\circ}$  in the oven to dehydrate the silica. The residue is moistened with a few drops of water and HCl, and again heated for 1 hr. at  $105^{\circ}$ .

The dehydrated silica is separated from the other constituents of the residue by bringing the latter into solution with dil. HCl. The residue is moistened with conc. HCl and the crucible three-quarters filled with water. The mixture is warmed on the steam bath and stirred with a glass rod. When solution appears to be complete, the crucible is removed from the bath and allowed to stand for a few minutes for the silica precipitate to settle.

Filtration of the silica is accomplished without removing it from the crucible by means of the filter stick illustrated in Fig. 1. In this way it is possible to complete all the operations involved in the analysis in the original vessel without

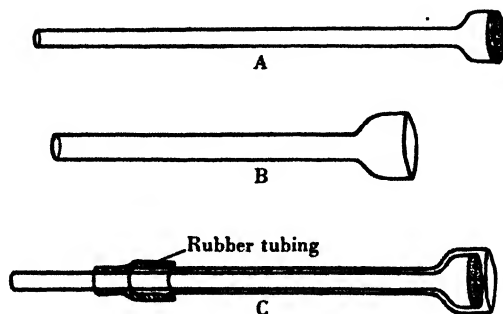


Fig. 1. Special filter stick for silica analysis. A, sintered glass filter stick. B, jacket. C, assembled filtering apparatus. (Obtainable from Messrs Gallenkamp.)

transferring the precipitate. This makes for greater accuracy in the method by avoiding the possibilities of error involved in transference. Filtration by sucking off the filtrate and washing is, moreover, faster than filtration by gravity. As a filtering medium, filter paper pulp shaken up with water, or powdered anthracene is suitable. The latter is easily eliminated by mild heating, whereas paper must be destroyed by ignition. The filtering medium is packed tightly into the cavity formed at the flared end of the filtering apparatus. A piece of fine rubber

suction tubing connects the filter stick to a small suction flask. Suction may be applied by means of a water pump, or, preferably, by mouth with a piece of rubber tubing carrying a pinch-cock attached to the side arm of the flask.

The filter stick is held so that its filtering surface is just below the level of the liquid in the crucible, and the filtrate sucked off under mild suction. The crucible is filled again with warm dil. HCl and the precipitate stirred, allowed to settle, and the supernatant again sucked off. Washing is repeated once with dil. HCl and twice with water. The precipitate is finally sucked dry in the bottom of the crucible.

The plug of filtering medium is dropped out of the filtering apparatus into the crucible by pushing the inner tube of the apparatus (the filter stick) a few mm. through the rubber collar which attaches it to the outer tube or jacket. Particles of silica adhering to the glass are washed into the crucible with alcohol or water from a wash bottle. Any liquid in the crucible is now evaporated on the water bath. The crucible is dried in an oven, or carefully with a flame, and then slowly ignited to red heat, cooled in a desiccator and weighed. HF (5 ml.) is added and a few drops of  $\text{H}_2\text{SO}_4$ . The crucible is gently heated to evaporate off the HF and  $\text{SiF}_4$ , and then more strongly to drive off the  $\text{H}_2\text{SO}_4$ . It is then ignited, cooled and weighed. The loss in weight on treatment with HF is taken as the weight of silica. Some typical results obtained by this method are given in Table I.

Table I. *Gravimetric analyses of silicotic tissues*

Tissue	Wt. of dry sample g.	mg. $\text{SiO}_2$ found	% $\text{SiO}_2$
Spleen 108 (1)	2.000	10.2	0.51
(2)	2.000	10.9	0.54
(3)	2.000	10.6	0.53
Lung 109 (1)	0.250	2.2	0.88
(2)	1.000	9.2	0.92
(3)	2.000	18.3	0.91
Lung 110 (1)	2.000	16.0	0.80
(2)	2.000	16.2	0.81
Lung 111 (1)	1.000	13.4	1.34
(2)	2.000	28.2	1.41
Lung 116	2.127	142.7	6.71
Lung 121 (1)	1.030	17.2	1.67
(2)	1.103	17.6	1.60

#### *The colorimetric determination of silica*

The colorimetric method adopted for the determination of silica in tissues consists in the production of a yellow silicomolybdic acid complex. This colour may be utilized as such, or it may be reduced by 1:2:4-aminonaphtholsulphonic acid to give a blue colour. Both colours are proportional to the amount of silica present [King, 1926; King & Stantial, 1933] and can be compared colorimetrically with those produced in standard solutions containing a known amount of silica.

The method is applicable to much smaller amounts of silica-containing material and can be executed in a fraction of the time that is necessary for gravimetric determinations. If the procedure is rigidly followed and due precautions are taken to insure the purity of the reagents used and to eliminate interfering substances, the results obtained are nearly as accurate as those given by the gravimetric procedure.

The yellow colour method is much less sensitive than the blue colour method. It may be used with any materials having a high silica content, such as silicotic tissue, lungs and lymph glands. The sensitive blue colour method may be used for any material, but is especially suitable for those of low silica content, normal tissues, blood, urine etc. The yellow silicomolybdic acid colour is preferably read in a photoelectric colorimeter, a photometer, or in Nessler or Hehner tubes. It is difficult to match in a Duboscq colorimeter. The reduced silicomolybdic acid blue colour can be assessed in any type of colorimeter; it is easily read in a Duboscq instrument. The comparison is greatly facilitated and made much more accurate if made in the light of a sodium lamp, such as those supplied with the Hellige and Klett instruments.

Several substances interfere with the reaction giving the silicomolybdic blue colour. Of these phosphate and iron are the most important in biological material. Phosphate gives a blue colour in the presence of molybdic acid and aminonaphtholsulphonic acid at all acidities. As phosphorus is an invariable constituent of animal tissues, its removal is necessary before the silica can be estimated. The presence of iron in the solution leads to the development of a greenish tint, and the colour can neither be matched against a simple silicate standard, nor is it proportional to the amount of silica present. In the original method the phosphate and iron were removed in two stages, the iron by precipitation with excess ammonium dihydrogen phosphate and the phosphate by precipitation with calcium chloride and ammonia.

The simultaneous elimination of phosphate and iron from a silicate-containing solution is accomplished by the basic ferric acetate procedure of Jacobs [1931], which was previously used for the preparation of a protein- and phosphate-free filtrate of blood. By this means the alkaline precipitation with ammonia is avoided and the precipitation of phosphate can therefore be carried out in glass vessels. Moreover, both phosphate and iron are precipitated in one step instead of the two which were previously necessary. The method removes phosphates as ferric phosphate, excess iron as basic ferric acetate and any colloidal or suspended material which may be present, such as protein, fat and debris (and in the case of urine, part of the pigment).

#### *Solutions and reagents*

*Silica standard. Sodium silicofluoride.* The most satisfactory standard used thus far is a solution of sodium silicofluoride. This salt is a light anhydrous non-hygroscopic powder which hydrolyses in solution to give silicic acid. 314 mg. of the solid in 1 l. of water give a concentration equivalent to 1 mg.  $\text{SiO}_2$  in 10 ml. ("strong standard"). A "weak standard" (0.1 mg.  $\text{SiO}_2$ /10 ml.) is made by diluting the "strong standard" 1 in 10. It has been found advisable to make these fresh each week.

10 N  $\text{H}_2\text{SO}_4$ . 278 ml. of conc. acid to 1 l. with water.

N  $\text{H}_2\text{SO}_4$ . 1 in 10 dilution of 10 N.

N  $\text{NaOH}$ . The  $\text{NaOH}$ , to be silica-free, must be prepared from the metal. 2.3 g. metallic sodium are dissolved in 100 ml. of water in a nickle crucible, and preserved in a wax bottle. B.D.H. "sodium hydroxide pure (from sodium)" is satisfactory. Glass pipettes should be brought into the  $\text{NaOH}$  solution only for the briefest possible times.

*Ferric chloride* in hydrochloric acid. 1 %  $\text{FeCl}_3$ ,  $6\text{H}_2\text{O}$  in 0.02 N  $\text{HCl}$ .

*Sodium acetate* in sodium hydroxide. 1.5%  $\text{C}_2\text{H}_3\text{O}_2\text{Na}$ ,  $3\text{H}_2\text{O}$  in 0.028 N  $\text{NaOH}$ . The ferric chloride and sodium acetate solutions, when mixed in equal amount, diluted with water, heated and filtered, yield a fluid of approximately pH 6.

*Acid molybdate.* 5 % ammonium molybdate in  $N$   $H_2SO_4$ .

*Reducing agent.* 0.2 % 1:2:4-aminonaphtholsulphonic acid in 2.4 %  $Na_2SO_3$ , 7  $H_2O$  and 12 %  $NaHSO_3$ .

*Ammonium phosphate solution.* 2 %  $(NH_4)H_2PO_4$ .

*Calcium chloride.* 2 %  $CaCl_2$ .

*Perchloric acid.* 60 %  $HClO_4$ .

*Acetic acid.* Glacial and 10 %.

*Potassium ferrocyanide.* 5 % aqueous solution.

*Pyramidon.* 1 % aqueous solution.

The original article should be consulted for details of the preparation of solutions, and for the precautions which must be observed in the use of the method. Frequent "blank" determinations should be carried out on the several solutions to determine that they are silica-free. Only chemical reagents of analytical grade should be used.

The preservation of fluids for analysis should always be in non-glass vessels. Urine and blood which are kept in glass increase in silica content. The dissolution of silica from the glass can be retarded by the addition of acid to the fluid; this is only possible in the case of urine. The Lusteroid test-tubes made of heavy cellophane by the International Equipment Co. of Boston, U.S.A. (and sold by Messrs Gallenkamp) are satisfactory for the collection and preservation of fluids for silica analyses.

*Tissue analysis:* 0.1–0.5 g. (depending on its silica content) of dry tissue is weighed into a platinum crucible or dish (preferably of large size, 25–50 ml.). Anhydrous  $Na_2CO_3$  equal to about four times the weight of tissue is added and the mixture carefully heated. After the sample has been "smoked off" destruction of the remaining organic matter takes place smoothly and rapidly in the melting mass. The heating is continued until a clear melt is obtained. Water (about 10 ml.) is added to the cooled crucible, and the fused material dissolved by heating on the steam bath. When solution is complete the crucible is cooled, and its contents washed through a funnel into a 100 ml. volumetric flask. The solution is carefully neutralized with  $N$   $H_2SO_4$ , using Congo red<sup>1</sup> as indicator, and shaken vigorously the while to rid the mixture of  $CO_2$ . The titration is facilitated by first neutralizing to phenolphthalein with 10  $N$   $H_2SO_4$  and then finishing the titration with Congo red and  $N$   $H_2SO_4$ . Approximately the same amount of acid is necessary for the second part of the titration as for the first. 20 ml. each of ferric chloride and sodium acetate solutions are now added. The volume is adjusted to 100 ml., the mixture shaken and poured into a 250 ml. conical flask. It is brought rapidly to the boiling point with vigorous shaking. By this means the iron and phosphate are completely removed as ferric phosphate and basic ferric acetate. The hot mixture is poured on a folded 30 or 32 Whatman filter paper. A water-clear filtrate is obtained which is suitable for the colorimetric silica estimation.

In order to determine that the solution is now phosphate-free, 1 ml. of the cooled filtrate should be tested for  $PO_4^{3-}$  ion by the addition of 2 drops of 10  $N$   $H_2SO_4$ , 2 drops of molybdate and 1 of reducing agent. The development of any blue colour indicates the presence of phosphate, and the basic ferric acetate precipitation should be repeated by heating 50 ml. of the filtrate with 10 ml. of ferric chloride and of sodium acetate.

Congo red is preferred as indicator because it is completely removed in the subsequent precipitation with basic ferric acetate. Other indicators, e.g. methyl orange, are not so removed, and make the final colorimetry difficult or impossible.

A second 1 ml. portion of the filtrate is tested for iron by the addition of 2 drops of potassium ferrocyanide solution and a drop of dilute acetic acid. Careful neutralization of the solution before the ferric acetate treatment usually ensures that no phosphate or iron will come through in the filtrate.

#### *Yellow colour method*

An aliquot portion of the filtrate—10, 25 or 50 ml. according to the amount of silica expected—is placed in a 50 ml. flask or cylinder. For standards 2, 5 and 10 ml. of "strong standard" (0.2, 0.5 and 1.0 mg.  $\text{SiO}_2$ ) are transferred to 50 ml. flasks. The volumes of tests and standards are adjusted to 50 ml., and 4 ml. of acid molybdate are added to each. The yellow colours are compared after 10 min. in Nessler or Hehner tubes, or in a photometer or photoelectric colorimeter.

#### *Blue colour method*

An amount of the filtrate which may be expected to contain from 0.02 to 0.12 mg.  $\text{SiO}_2$  is transferred to a 25 ml. volumetric flask. 2, 5 and 10 ml. portions of the "weak standard" (equivalent to 0.02, 0.05 and 0.1 mg.  $\text{SiO}_2$ ) are pipetted into similar flasks. Water is added to about 20 ml., and then 2 ml. of acid molybdate and 0.5 ml. of reducing agent. The flasks are shaken, and kept for 10 min. for the colours to develop. 10N  $\text{H}_2\text{SO}_4$  (1 ml.) is then added to each flask to stabilize the blue colours, the volumes are adjusted to 25 ml. and the contents of the flasks thoroughly mixed. Comparison of the test is then made against the appropriate standard.

*Notes on tissue analysis.* Lung and thoracic and mesenteric lymph nodes of adults are usually much richer in silica than other tissues and small samples should be used for analysis. 100 mg. (or less) of a silicotic lung powder or of peribronchial and mediastinal lymph nodes give sufficient silica for colorimetric determinations (lung tissue and lymph glands from young persons or animals however require the larger amounts).

Brain and nerve tissues have higher phosphate contents than most other tissues and here 25 ml. of the precipitating reagents should be used. (Sufficient of the reagents should be added before boiling to provide an excess of iron which is indicated by the formation of the brown precipitate of basic ferric acetate. If insufficient iron is present only white ferric phosphate is formed and the filtrate will contain at least a trace of phosphate when tested.)

Bone, which is largely calcium phosphate, is difficult to ash, and more sodium carbonate is necessary, the melt never becoming clear. After the fusion mixture has been taken up in water and neutralized, the precipitate of calcium phosphate should be removed by filtration, the precipitate and filter paper being washed thoroughly to remove all silica. The filtrate still contains much phosphate and 25 ml. each of the ferric chloride and sodium acetate precipitating reagents may be required.

Calcified lymph nodes present the same difficulties as bone and should be treated in a similar manner.

A comparison of results obtained by the yellow and blue colour methods and by gravimetric analysis is given in Tables II and III.

Table II. *Comparison of silica analyses by different methods*

Tissue	Blue colorimetric % $\text{SiO}_2$	Gravimetric % $\text{SiO}_2$
Spleen 108, abrasive soap powder worker	0.54	0.53
Lung 109, silica brick grinder	0.85	0.90
Lung 110, coal miner	0.82	0.80
Lung 112, coal miner	1.45	1.45
Lung 116, fuller's earth worker	6.70	6.71
Lung 121, coal miner	1.34	1.53

Table III. *Comparison of results of tissue analyses by yellow and blue colorimetric procedures*

Lymph glands	Age in years	Yellow colour method	Blue colour method
228, housewife	43	0.98	0.98
257, traveller	60	1.33	1.34
259, labourer	41	1.23	1.26
271, housewife	73	1.37	1.37
275, housewife	86	1.78	1.75
286, pensioner	79	0.64	0.63
289, housewife	46	1.07	1.03
290, taxi-driver	61	0.93	0.95
293, laundress	50	1.05	1.00
394, policeman	80	0.64	0.64
582, motor driver	67	0.61	0.62
586, housewife	73	2.38	2.41
597, housewife	87	1.58	1.57
601, dairyworker	44	1.33	1.35
606, housewife	62	3.27	3.33
643, pensioner	74	1.37	1.35
663, hairdresser	62	0.53	0.54

*Urine analysis*

5 ml. of human urine (or of the urine of carnivorous animals) are treated with 5 ml. of water and 20 ml. each of ferric chloride and sodium acetate solutions. 1 or 2 ml. of the urine of herbivorous animals, which contains a much higher concentration of silica, are sufficient. The mixture is heated rapidly to boiling in a conical flask, and poured hot on the filter paper.

The filtrate should be clear and practically colourless. It is tested for phosphate and iron. If either be found, a fresh specimen of urine should be carefully acidified with acetic acid (Congo red indicator) and the precipitation repeated. 10 or 20 ml. of filtrate are transferred to 25 ml. volumetric flasks, and 2, 5 and 10 ml. portions of "weak standard". The solutions are treated as previously described to develop the blue colours.

Table IV. *Colorimetric analysis of urine—recovery of added silica*

	mg. SiO <sub>2</sub> per 100 ml.	SiO <sub>2</sub> added, mg. per 100 ml.	Total SiO <sub>2</sub> found, mg. per 100 ml.	mg. SiO <sub>2</sub> recovered
Synthetic urine	0	0.5	0.5	—
" "	0	3.0	2.98	—
Dog urine	0.53	1.0	1.47	0.94
Sheep urine	17.2	5.0	22.1	4.9
Human urine	0.85	1.0	1.87	1.02
" "	1.56	1.0	2.63	1.07
" "	2.35	1.0	3.32	0.97
" "	2.35	5.0	7.40	5.05
Human urine after ingestion of silicic acid	6.85	1.0	7.87	1.02
	6.85	10.0	17.0	10.15

*Blood analysis—soluble silica*

*Perchloric acid—basic ferric acetate deproteinization.* "Direct" method. The deproteinization of blood with basic ferric acetate, which was previously described [King & Stantial, 1933], has not in practice always yielded a completely protein-free filtrate. For this reason a preliminary partial deproteinization with perchloric acid is now recommended. The phosphate in the filtrate is then removed by the basic ferric acetate method. The resulting filtrate contains all the

silica originally present in the blood, as is shown by comparison of the values obtained by this method with those for ashed samples of blood. Measured amounts of silicic acid, added to the blood, have been quantitatively recovered in the filtrate by the colorimetric procedure.

2 ml. of blood (fresh or oxalated—1 drop of 10 % potassium oxalate for 3 ml. blood) are laked with 10 ml. of distilled water. 1 ml. of calcium chloride solution and 0.3 ml. of perchloric acid (60 %) are added. The mixture is shaken and centrifuged. 10 ml. of the supernatant are transferred to a 15 ml. volumetric flask. A drop of phenolphthalein is added, and *N* NaOH to neutralize the perchloric acid. The pink colour is discharged and the solution made faintly acid by a small drop of glacial acetic acid. 1 ml. each of ferric chloride and sodium acetate are added and the volume adjusted to 15 ml. The mixture (transferred to a 50 ml. conical flask) is brought rapidly to the boil with vigorous shaking over a free flame, and is poured, while still hot, on a folded filter.

10 ml. of cooled filtrate (equivalent to 1 ml. of blood) with 1.2 ml. of molybdate and 0.3 ml. of aminonaphtholsulphonic acid are transferred to a 15 ml. volumetric flask for the colorimetric estimation. A 0.01 mg.  $\text{SiO}_2$  standard is used.

*"Indirect" method by precipitation with pyramidon.* King & Watson [1936] found that silica could be completely precipitated from solution as the pyramidon salt of silicomolybdic acid. Advantage of this fact may be taken to concentrate the blue colour in a smaller volume, thereby rendering it more easily assessable in the colorimeter. 10 ml. of filtrate are treated in a conical 15 ml. centrifuge tube with 1 ml. of acid molybdate and 1 ml. of pyramidon solution. The precipitate of pyramidon silicomolybdate is allowed to flocculate during 10 min. in a beaker of water at about  $60^\circ$ , and is then centrifuged down at 3000–4000 r.p.m. for 10 min. The supernatant is carefully poured off and the tube inverted to drain on filter paper for 5 min. The precipitate is dissolved in 0.5 ml. of reducing agent, and diluted with 3 ml. of water and 0.5 ml. of acid molybdate. Standards consisting of 1 ml. of "weak standard" and of 0.5 ml. with 0.5 ml. of water are precipitated with 2 drops each of sodium acetate, ammonium molybdate and pyramidon.

The final 4 ml. of coloured solution obtained by this procedure are of a much more intense blue than the 15 ml. of coloured solution obtained from the same amount of blood in the "direct" method. Because of the small volume of coloured solution it is necessary to use a colorimeter equipped with micro or semi-micro attachments.

Table V. *Colorimetric analyses of blood, recovery of added silica*

	mg. $\text{SiO}_2$ per 100 ml. of blood	mg. $\text{SiO}_2$ added per 100 ml.	Total mg. found per 100 ml.	mg. $\text{SiO}_2$ recovered
Horse serum	1.18	2.0	3.23	2.05
" "	1.18	3.0	4.11	2.93
Defibrinated horse blood	1.27	3.0	4.16	2.89
" " "	1.31	3.0	4.16	2.85
Mixed, oxalated human blood (1)	1.66	1.0	2.60	0.94
(2)	1.47	1.0	2.50	1.03
(3)	{ 1.39 1.38	{ 1.0 1.0	{ 2.22 2.25	{ 0.83 0.87
(4)	{ 1.14 1.14	{ 2.0 3.0	{ 2.91 4.28	{ 1.77 3.14
(5)	1.30	3.0	3.96	2.66
(6)	1.39	3.0	4.42	3.03



*Blood serum* may be treated as in the perchloric acid-basic ferric acetate method for whole blood.

The bloods used in the recovery experiments (Table V) and for comparison of methods (Table VI) were preserved with the ordinary grade of potassium oxalate in the soft glass containers used for the routine collection of blood in the hospital. It will be noted that the figures are higher than those shown in Table VII for samples of blood preserved with analytical grade potassium oxalate, or with heparin, in non-glass vessels.

Table VI. *Comparison of "direct" and "indirect" methods for blood*

	mg. of SiO <sub>2</sub> found per 100 ml. of blood	
	"Direct" method (HClO <sub>4</sub> -FeOAc precipitation of proteins)	"Indirect" method (pyramidon-molybdate precipitation of SiO <sub>2</sub> from filtrate)
Oxalated human blood	1.30	1.39
	1.27	1.31
	0.93	1.00
	1.06	1.12
	2.63	2.63
	1.50	1.42
	1.42	1.47
	1.03	1.13
Blood to which sodium silicate has been added	2.10	2.17
	2.69	2.73
	4.16	4.16
	3.76	3.94
	3.48	3.61

Table VII. *Silica of human blood*

	mg. SiO <sub>2</sub> per 100 ml. of blood	
	1.11	1.13
Blood preserved in glass	0.89	0.90
" "	0.84	0.84
Fresh blood (post-prandial)	0.47	0.45
" (1 hr. post-prandial)	0.45	0.46
" (3 hr. " " )	0.43	0.42
Heparinized blood, in cellophane tubes	0.62	0.63
" " "	0.53	0.55
" " "	0.30	0.33
Oxalated blood, in cellophane tubes	0.60	0.62
" " "	0.32	0.30
" " "	0.42	0.35

#### *Ascitic fluid and C.S.F.*

Fluids such as these, of low protein content, are best treated by a direct deproteinization (with simultaneous elimination of phosphate) with basic ferric acetate [King & McGeorge, 1938]. 3 ml. portions of the fluid are treated in 100 ml. conical flasks with 6 ml. of ferric chloride and 6 ml. of sodium acetate solution. The mixtures are brought quickly to the boil, with vigorous shaking, and filtered hot. 10 ml. of filtrate in 15 ml. flasks are treated as for blood filtrates.

*Total silica of blood*

5 ml. of blood with 1 ml. of ammonium phosphate solution<sup>1</sup> are carefully dried in a platinum crucible. 0.5 g.  $\text{Na}_2\text{CO}_3$  is used for the ashing and the analysis carried out as in the case of "Tissue Analysis".

*Sputum analysis*

Because of the presence of mineral particles in the sputa of individuals who have inhaled siliceous dust [cf. Burke, 1935], the analysis of the sputum for silica may occasionally be desirable. The analysis is best carried out on a weighed sample; because of its viscous nature sputum is not conveniently measured with a pipette. The samples are collected in waxed paper containers, and poured into weighed platinum crucibles. The wet weight is recorded, and the dry weight after drying at  $105^\circ$ .  $\text{Na}_2\text{CO}_3$  is added, the sample is ashed, and the analysis completed as for tissues. Results are probably better expressed as a percentage of the dry weight, since the water content of sputum varies greatly.

*Determination of silica in mineral dusts*

It is frequently necessary, in investigations related to silicosis, to analyse samples of rock or of rock-dust for their total silica content. The gravimetric analysis will usually be preferred, but in special circumstances, e.g. with small samples of air-borne dust, the colorimetric procedure may be necessary. If the mineral specimen is known to contain no phosphorus and no iron, it should be fused with  $\text{Na}_2\text{CO}_3$ , dissolved in water, brought to an appropriate volume and a suitable amount taken for colorimetric analysis. Most industrial dusts, however, contain iron. Ferric silicate is insoluble in an alkaline medium, viz. the solution of the  $\text{Na}_2\text{CO}_3$  melt, and although hydrolysed by acid it may not be recovered on neutralization of the  $\text{Na}_2\text{CO}_3$  solution because of its sticking to the walls of the platinum crucible. In order to avoid loss of silica through precipitation as iron silicate it is necessary to add sufficient phosphate to insure the complete removal of the iron as insoluble ferric phosphate. The excess phosphate is removed in the subsequent treatment with ferric chloride and sodium acetate.

An amount of mineral dust sufficient to contain 1–10 mg.  $\text{SiO}_2$  is fused with  $\text{Na}_2\text{CO}_3$ . 1 ml. of phosphate solution is added and water to dissolve the melt. The subsequent procedure is the same as for tissues. Some results are shown in Table VIII.

Table VIII. *Analysis of mineral dusts. Colorimetric method*

	Weight of sample mg.	% $\text{SiO}_2$ found	% $\text{SiO}_2$ found by gravimetric analysis
Quartz	10	97.0	96.6
Kieselguhr	5	87.7	88.3
	50	88.2	—
Flint	10	90.1	89.0
Stone dust	5	52.3	54.9

<sup>1</sup> See the section on "Mineral Dusts", in connexion with the necessity of adding extra phosphate in the case of materials containing an excess of iron over the phosphorus present.

## SUMMARY

Methods for the determination of silica in tissues and body fluids are described. Directions are given for a gravimetric procedure using a modified filter stick, and for colorimetric procedures which employ the silicomolybdic acid yellow colour and the reduced silicomolybdate blue colour.

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# CXVII. INVESTIGATIONS ON THE POLYPEPTIDE-CONTENT OF THE SERUM

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EXISTING methods for determination of the polypeptide content of serum are mainly based on two different principles. According to the first the protein is removed and estimations are made with the protein-free filtrates. It is thus essential that the protein precipitant should not precipitate any polypeptides. In such a protein-free filtrate the amino-nitrogen may be determined before and after hydrolysis, the difference is taken as an index for the polypeptide content [Hiller & Van Slyke, 1922; Becher & Herrmann, 1931, 1, 2; 1932, 1, 2; Martens 1930].

In the second type of method two different protein-free filtrates of the serum are made, of which the first contains the polypeptides whereas the second filtrate does not. The difference in some chemical constituent is taken as an index of the polypeptide content, e.g. the nitrogen content [Hahn, 1921; Wolff, 1921; Puech & Cristol, 1929] or the tyrosine content [Goiffon & Spaey, 1934].

Both principles have been strongly criticized. It is the object of this paper to demonstrate that under the correct circumstances reliable results may be obtained with the aid of a method based on the second.

## *Choice of method*

The method used was that of Goiffon & Spaey, the proteins being first removed from the serum with the aid of two different protein precipitants.

The first filtrate is obtained by mixing 1 vol. serum with 9 vol. 3% trichloroacetic acid; after standing for at least 15 min. the solution is filtered. It will be shown that this solution still contains the polypeptides. The second filtrate is obtained by mixing 1 vol. serum with 9 vol. of a solution containing phosphotungstic acid (22 g.) and *N* HCl (30 ml.) in 1 l.; again after at least 15 min. the solution is filtered. This time the filtrate will not contain the polypeptides. Hence the filtrates will have different nitrogen and tyrosine contents, and the difference between the nitrogen content of the trichloroacetic acid filtrate (TN) and that of the phosphotungstic acid filtrate (PWN) will correspond to the nitrogen content of the polypeptides, the so-called "double nitrogen" (DN). 5 ml. of each filtrate are treated with two drops of the phenol reagent of Folin and Denis<sup>1</sup> and 4 ml. of 10%  $\text{Na}_2\text{CO}_3$ ; after 30–60 min. one drop of saturated aqueous  $\text{Na}_2\text{SO}_3$  is added and the volume made up with water to 10 ml. Comparison is made with 5 ml. of a standard solution of tyrosine, containing 0.01 g. tyrosine in 1 l. of 0.1 *N* HCl similarly treated. From the difference in colour the

<sup>1</sup> This reagent was prepared by dissolving 25 g. of sodium molybdate and 100 g. of sodium tungstate in 700 ml. of water; 50 ml. of 85% phosphoric acid and 100 ml. of 38% hydrochloric acid were added; after boiling for 8 hr. under reflux the solution was cooled and the volume adjusted to 1 l.

tyrosine index may be calculated which indicates the number of mg. of tyrosine contained in the polypeptides of 1 l. of serum. In order to prove that the double nitrogen and the tyrosine index really do depend on the presence of polypeptides, a peptidase (erepsin) was added to a number of sera; by this means the values of the double nitrogen and of the tyrosine index were reduced almost to zero. Care had to be taken that the erepsin preparation was free from proteases.

In the first experiments a commercial erepsin was used which proved useless since the preparation itself gave a strong colour with the reagents used for the determination of the tyrosine index; moreover, a few hours after its addition the serum proteins began to be broken down. The commercial preparation itself contained nitrogen compounds which were not precipitated by trichloroacetic acid, but were precipitated by phosphotungstic acid.

Erepsin was therefore prepared according to Waldschmidt-Leitz & Schäffner [1926]. 100 g. of the mucosa of the small intestine of the pig were ground up with 500 ml. of 87% glycerol. For each experiment 10–15 ml. of this suspension were mixed with 3 vol. water and centrifuged. The supernatant fluid, containing the erepsin, was precipitated with about 2 ml. 0.1 *N* acetic acid. The tube was inverted in order to remove the fluid as completely as possible. The precipitate was mixed with the serum, of which TN, PWN, DN and the tyrosine-index (TI) had just been estimated. The precipitate soon settled down. Estimations of DN and TI were carried out immediately and after several hours at 37° [Godfried, 1938]. Table I shows some of the results.

Table I

Exp. no. ...	5			6			7			8		
	Serum + erepsin			Serum + erepsin			Serum + erepsin			Serum + erepsin		
	Serum	At once	After 18 hr.	Serum	At once	After 10 hr.	Serum	At once	After 10 hr.	Serum	At once	After 10 hr.
TN	51.2	50.7	50.1	56.3	56.3	56.3	45.1	45.6	46.8	59.1	59.2	59.9
PWN	30.0	30.0	50.1	50.7	50.2	55.8	38.1	37.6	46.2	57.2	57.3	59.9
DN	21.2	20.7	0	5.6	6.1	0.5	7.0	8.0	0.6	1.9	1.9	0
TI	38	36	2	18	18	2	18	18	0	—	—	—

Exp. no. ...	9			10				11		
	Serum + erepsin			Serum + erepsin				Serum + erepsin		
	Serum	At once	After 3 hr.	Serum	At once	After 6 hr.	After 30 hr.	Serum	At once	After 5 hr.
TN	18.5	18.6	18.5	143.1	142.0	143.1	142.5	31.1	36.7	37.5
PWN	13.2	13.4	18.5	116.8	116.8	124.3	140.6	24.9	30.5	36.1
DN	5.3	5.2	0	26.3	25.2	18.8	1.9	6.2	6.2	1.4
TI	22	22	0	46	46	32	4	26	26	0

TN = trichloroacetic acid filtrate-N; PWN = phosphotungstic acid filtrate-N; DN = polypeptide-N all in mg./100 ml.; TI = tyrosine index.

From these experiments the conclusion may be drawn that the double nitrogen and the tyrosine index correspond to polypeptides which are broken down by erepsin.

In a further experiment glycyl-*L*-tyrosine and *D*-leucylglycine were added to serum; the nitrogen of these compounds could be recovered quantitatively in the trichloroacetic acid filtrate, but was absent from the phosphotungstic acid filtrate.

The results obtained by the tyrosine method did not always run parallel with those obtained by the nitrogen estimations, presumably owing to the variability of the tyrosine and nitrogen contents of different polypeptides.

### *Results*

According to the tyrosine method the normal value of the polypeptide content of the serum is less than 25 (as mg. of tyrosine per litre of serum); according to the nitrogen method, described above, it is less than 7 mg./100 ml.

Conditions in which the serum content of polypeptides is increased may be divided into three groups. Some of the results are shown in Table II. For more detailed figures the reader is referred to the author's thesis [Godfried, 1938].

The first group contains those cases in which there is increased parenteral destruction of proteins. In gastric and duodenal ulcer hyperpolypeptidaemia has been reported [Marino & Saladino, 1937]; in the writer's experience such hyperpolypeptidaemia was only found shortly after a haemorrhage. Suppuration may be accompanied by hyperpolypeptidaemia [Hülse & Strauss, 1924]. This has been confirmed in the present work and attention is drawn to the increased content of polypeptides in ulcerative colitis. When the condition of a patient suffering from ulcerative colitis improves, the hyperpolypeptidaemia becomes less marked (Table II, no. V<sup>1</sup>). In leukaemia, especially when the patient has been treated with X-rays, hyperpolypeptidaemia may be marked. In malignant new growth, chiefly when extensive metastases exist, very high indices for the polypeptide content may be found. The highest values are reached when metastases have formed in the liver. Among diseases of metabolism the presence of hyperpolypeptidaemia in severe diabetes [Puech, 1926, 1, 2] has been confirmed. In several cases of sprue and of gout an increase in serum polypeptides was also found.

The second group comprises diseases of the liver. It is well known, that under normal conditions polypeptides are broken down to urea or synthesized into proteins in the liver [Fiessinger, 1934]. In cirrhosis of the liver hyperpolypeptidaemia may or may not be found [Labbé & Nepveux, 1931<sup>1</sup>; Becher & Herrmann, 1931, 1, 2; 1932, 1, 2; Valdigué, 1934; Bentz & Larizza, 1937]. Reference has already been made to the association of malignancy involving metastases in the liver with a high polypeptide content of the serum; the estimation of the degree of the polypeptidaemia in jaundiced patients may indeed be of great value, especially to assess the indication for cholecystogastrostomy in cases of a carcinoma of the head of the pancreas. If such a carcinoma is still small, it may nevertheless cause jaundice; in such cases cholecystogastrostomy may enable the bile to flow away from the liver along the intrahepatic bile passages. If, however, large metastases have formed in the liver, these will obstruct the intrahepatic bile passages and cholecystogastrostomy will not cause the disappearance of the jaundice. If hyperpolypeptidaemia is found in cases of carcinoma of the pancreas, the presence of large metastases is very probable and cholecystogastrostomy will remain without any effect. Cases IX<sup>32</sup> and IX<sup>33</sup> demonstrate this clearly; in both patients, suffering from a carcinoma of the head of the pancreas and of the bile passages respectively, marked jaundice and hyperpolypeptidaemia were present; in both cholecystogastrostomy was performed, but in both it remained without any result. However, even if metastases in the liver exist in a jaundiced patient suffering from a carcinoma of the head of the pancreas, the polypeptide content of the serum may still be low (compare case IX<sup>30</sup>).

The third group is formed by diseases of the kidneys [Pribram & Klein, 1923; Becher & Herrmann, 1931, 1, 2; 1932, 1, 2; Puech & Cristol, 1929; Martens, 1928; Larizza, 1937]. High blood pressure alone is not accompanied by a hyperpolypeptidaemia; acute and chronic nephritis, however, are; apparently the diseased kidney does not excrete sufficient polypeptides. No parallelism exists between the polypeptide content and the non-protein-nitrogen. The polypeptide content of the serum in a patient suffering from chronic nephritis does not give any prognosis of the duration of life, nor does any relation exist between the polypeptide content and the usual kidney function tests (phenosulphophthalein-test, urea clearance etc.). The raised values in cases of cardiac decompensation may be considered to be caused by disturbance of the functions of the liver and of the kidneys.

Table II. *Polypeptide content of serum in various diseases*

No.	Sex	Age	Diagnosis	TI	TN	PWN	DN
III <sup>2</sup>	♂	39	Duodenal ulcer	42	—	—	—*
				22	—	—	—†
III <sup>8</sup>	♂	35	Haemorrhage from a gastric ulcer	52	—	—	—
IV <sup>2</sup>	♂	69	Pneumonia	50	—	—	—
IV <sup>6</sup>	♂	30	Bronchiectasis	54	—	—	—
IV <sup>8</sup>	♂	24	Abscess of the lung	62	32.5	9.8	22.7
V <sup>1</sup>	♂	28	Ulcerative colitis	70	—	—	—
				30	35.6	28.8	6.8‡
V <sup>2</sup>	♂	26	Do.	42	—	—	—
V <sup>3</sup>	♂	11	Do.	104	—	—	—
V <sup>9</sup>	♂	23	Do.	46	22.4	11.5	10.9
V <sup>12</sup>	♂	35	Do.	44	71.4	61.3	10.1
V <sup>13</sup>	♂	51	Do.	44	33.0	16.2	16.8
VI <sup>2</sup>	♂	49	Lymphatic leukaemia	34	63.6	54.0	9.6
VI <sup>5</sup>	♂	50	Do.	86	—	—	—§
VII <sup>4</sup>	♂	64	Tumour of the lung	128	—	—	—
VII <sup>8</sup>	♂	59	Metastases of a carcinoma of the chest	36	29.7	18.7	11.0
VIII <sup>1</sup>	♂	47	Diabetes	80	—	—	—
VIII <sup>2</sup>	♂	40	Do.	88	—	—	—
VIII <sup>12</sup>	♂	53	Gout	80	—	—	—
VIII <sup>5</sup>	♂	53	Sprue	68	—	—	—
VIII <sup>7</sup>	♂	65	Do.	40	30.5	20.4	10.1
VIII <sup>9</sup>	♂	51	Do.	—	51.8	30.0	21.1
IX <sup>2</sup>	♂	57	Cirrhosis of the liver	100	—	—	—
IX <sup>9</sup>	♂	67	Cirrhosis of the liver; gall stones	44	23.0	11.8	11.2
IX <sup>11</sup>	♂	26	Do.	4	24.9	23.2	1.7
IX <sup>12</sup>	♂	53	Do.	10	27.4	25.8	1.6
IX <sup>24</sup>	♂	40	Gall stones; abscess of the liver	56	—	—	—
IX <sup>26</sup>	♂	52	Metastases of a carcinoma of the chest	120	—	—	—
IX <sup>20</sup>	♂	48	Carcinoma of the head of the pancreas; metastases in the liver	30	—	—	—
IX <sup>21</sup>	♂	52	Do.	124	—	—	—
IX <sup>22</sup>	♂	59	Do.	80	—	—	—
IX <sup>23</sup>	♂	60	Carcinoma of the bile passages	138	—	—	—
X <sup>3</sup>	♂	46	Hypertension	16	26.6	22.4	4.2
X <sup>5</sup>	♂	54	Do.	26	31.1	24.9	6.2
XI <sup>8</sup>	♂	40	Acute nephritis	56	—	—	—
XI <sup>10</sup>	♂	17	Do.	40	31.1	21.8	9.3
XI <sup>24</sup>	♂	40	Chronic nephritis	50	160.4	144.7	15.7
XI <sup>26</sup>	♂	36	Do.	26	48.7	41.4	7.3
XI <sup>28</sup>	♂	60	Do.	38	21.6	9.5	12.1
XI <sup>24</sup>	♂	21	Do.	40	43.7	30.2	13.5
XI <sup>25</sup>	♂	25	Do.	46	143.1	116.8	26.3
XIII <sup>12</sup>	♂	46	Cardiac decompensation	78	73.9	53.7	20.2
XIII <sup>5</sup>	♂	30	Do.	88	—	—	—

\* Immediately after a haemorrhage.

† 18 days afterwards.

‡ A year after the first estimation.

§ Treated with X-rays.

|| See text.

## SUMMARY

It has been shown that the differences in nitrogen content or in tyrosine content of two filtrates of the same serum, obtained after the addition of 3% trichloroacetic acid and phosphotungstic acid respectively, correspond to the polypeptides of the serum. Hence these differences form a reliable index of the polypeptide content of the serum. The circumstances where the serum contains excessive amounts of polypeptides are discussed.

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# CXVIII. PORPHYRINURIC ACTION OF DRUGS RELATED TO SULPHANILAMIDE. COMPARISON WITH REPORTED TOXICITY, THERAPEUTIC EFFICIENCY AND CAUSATION OF METHAEMOGLOBINAEMIA. DEFINITION OF THE STRUCTURE RESPONSIBLE FOR PORPHYRINURIC ACTION

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It has been shown [Rimington & Hemmings, 1938] that a marked increase in porphyrin excretion follows the daily oral administration to white rats of sulphanilamide at dosage levels of 0.4–1.5 g./kg. body weight. The porphyrins were isolated from urine and faeces and identified as coproporphyrin III together with small quantities of coproporphyrin I. Porphyrinuria due to these same two pigments was demonstrated in a series of human clinical cases undergoing a course of sulphanilamide treatment, and similar observations have more recently been reported by Long & Bliss [1938], and also by Silver & Elliott [1939].

Discussion of the mechanism of this porphyrinuria led to the conclusion that the drug, in the doses employed, exerts a toxic action most probably affecting both the liver and the bone marrow. The possibility was indicated that some patients might prove to be more susceptible than others and it was suggested that determination of the amount of porphyrinuria might serve as a means of assessing the toxic action of sulphanilamide upon this phase of haematopoietic activity and of revealing individual idiosyncrasy. That sulphanilamide is capable of causing profound disturbances in the pigment metabolism of the body is evident from the frequency with which cyanosis accompanied by demonstrable methaemoglobinaemia has been reported. Thus, in a review of the types of toxic manifestation occurring in 408 patients exhibiting sulphanilamide intoxication, Long *et al.* [1939] noted cyanosis in 90–100%, whilst most observers agree that at least half of all their patients treated with the drug show some degree of cyanosis. There has been much discussion as to whether methaemoglobin is alone responsible, or whether products derived from sulphanilamide may also contribute to the discoloration. Some observers have gone so far as to assert that methaemoglobinaemia is of comparatively rare occurrence; on the other hand, careful investigations by Lockwood *et al.* [1938], Hartmann *et al.* [1938] and others have shown that a close correspondence can be traced between the degree of cyanosis and the quantity of spectrophotometrically determined methaemoglobin. It cannot be too greatly emphasized that in particular those toxic effects revealed by changes in the pigment economy and metabolism of the organism are deserving of much more thorough investigation than has hitherto been accorded them [compare Rimington, 1939].

In the present communication, experiments are described by means of which answers to the following questions have been sought:

(1) In treatment with the sulphonamide series of drugs, is porphyrinuric activity an invariable accompaniment of therapeutic effectiveness or does this symptom parallel toxicity to the animal as a whole (minimal acute fatal dose)?

(2) Which chemical grouping or portion of the molecule is responsible for porphyrinuric activity?

(3) Is there any relation between methaemoglobin formation (compare sulphanilamide cyanosis) and increased excretion of porphyrin?

A series of substances related to sulphanilamide or possessing certain of the structural elements of this drug have been fed over periods of 1, 2 or 3 weeks to rats maintained, as in the previous experiments, upon a sufficient, synthetic diet. The doses were varied to allow of comparison as to porphyrinuric effect, and in all cases when a toxic action was manifest, post-mortem histological examinations have been made. The results demonstrate that porphyrinuric action is closely associated with ability to produce methaemoglobinaemia and runs roughly parallel with toxicity as a whole.

### EXPERIMENTAL

#### *Technique of animal experiments*

Certain improvements in technique have been made since the publication of our first paper, and our present method will therefore be recorded in detail.

White rats weighing 150–200 g. were kept in groups of three in metabolism cages arranged for the separate collection of urine and faeces. These cages were made of metal, the funnel-shaped lower portion being carefully treated with molten paraffin wax at intervals of about a week, to prevent any possibility of the urine coming into direct contact with the metal surface. Toluene was added to the glass urine-collecting flasks.

The artificial diet was made up as follows, and was fed *ad lib.* Water was supplied *ad lib.* from Drummond bulbs:

*Synthetic diet.* Casein, 20%; rice starch, 73%; salt mixture, 2%; yeast powder, 5%; cod-liver oil, 20  $\mu$ l. per rat per week.

*Salt mixture.* NaCl, 41.6 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 130.2 g.;  $\text{NaH}_2\text{PO}_4$ , 83.2 g.;  $\text{K}_2\text{HPO}_4$ , 228.8 g.;  $\text{CaHPO}_4$ , 129.6 g.; ferric citrate, 47.2 g.; calcium lactate, 146.2 g.

The food was not placed in the metabolism cages, but the animals were removed and allowed to feed and drink in separate cages for 1 hr. in the morning and 1 hr. in the evening (a single period of 2 hr. on Sundays). A small quantity of urine was unavoidably lost during this time, but contamination of the collecting receptacle with debris was obviated. All animals were weighed at weekly intervals. The drugs were administered orally, unless otherwise stated, usually in the form of a suspension in 10% gum acacia solution. A requisite volume of this mixture was introduced daily into the oesophagus by means of a hypodermic syringe fitted with a blunted metal needle. Liquid drugs were introduced into the oesophagus in the same manner. Groups of normal rats, kept under similar conditions, served as controls.

#### *Determination of urinary porphyrin*

The urine sample, representing a 3-day collection from three rats (about 70 ml.), was filtered through glass wool into a separating funnel and the collecting flask rinsed with 20–30 ml. of water. To the combined filtrates were added 20 ml. of glacial acetic acid followed by 200 ml. of ether. The mixture was well shaken and the lower aqueous layer was run off as far as the small

gelatinous emulsion which is almost invariably found at the interface. Half an hour was allowed for this emulsion to contract into a more solid mass, from which the ether was decanted. The emulsion residue was now shaken again with 3–4 ml. of acetic acid and 100 ml. of ether, this solvent, after decantation, being used for a final extraction of the main bulk of the urine. The combined ether extracts were washed four times with 200 ml. of water, the first two washings containing 5 ml. of saturated potassium acetate solution adjusted, if necessary, to pH 7.0. After removal of the bulk of the dissolved acetic acid from the ether phase in this way, the porphyrins were extracted by shaking with successive lots of 10 ml. of 5% (by weight) HCl, allowing the acid phase to settle down completely each time before tapping off. Completion of extraction can be controlled by spectroscopic or fluoroscopic examination. Usually three or four extractions suffice to remove all the porphyrin. Such an acid extract will only contain the porphyrin in very great dilution and may also contain other colouring matters which interfere with quantitative determination, especially if a fluorescence method be used. It has been our practice, therefore, to purify and concentrate the porphyrin as follows.

To the 5% HCl extract in a smaller separating funnel are added 5 ml. of ether and sufficient saturated potassium acetate to render the reaction neutral to Congo red. (8 ml. are sufficient for 30 ml. of acid extract.) After vigorous shaking, the ether is transferred to a 20 ml. capacity funnel having a very short outlet tube, and the aqueous solution is extracted with two more successive 5 ml. lots of ether, which are combined with the first. This ether solution is washed once with 3 ml. of water, taking care to shake down drops adhering to the glass. The coproporphyrin is then extracted by shaking with 0.5 ml. lots of 0.5% by weight HCl until the acid absorption spectrum is no longer visible. Usually five shakings suffice to remove even as much as 100  $\mu$ g. The volume is then carefully adjusted to 3 ml. (or more, if much porphyrin is present) and the quantity of coproporphyrin present determined by measuring the light absorption in the region of the  $\beta$  band of the acid porphyrin spectrum (centre about 549  $m\mu$ .) on a Hilger-Nutting spectrophotometer previously calibrated against standard coproporphyrin solutions. Since the relation between porphyrin concentration and the photometer scale reading is linear, the exact concentration of porphyrin in the test solution is readily obtained. Concentrations as low as 3  $\mu$ g./ml. in the final solution are easily determined by this method.

It will be noticed that we have employed 0.5% HCl for the final extraction of the coproporphyrin. The advantage of this procedure is that any protoporphyrin present through accidental contamination of the urine specimen with faeces is thereby left behind in the ether, since protoporphyrin has a much higher acid number than has coproporphyrin.

The rats selected for any particular experiment were usually allowed to remain for 6–12 days upon the basal synthetic diet, whilst a "base line" of porphyrin excretion was being obtained. Daily dosing was thereafter commenced and continued for 9–24 days, according to the promptness of response. In many instances, the effect did not become very marked until about the ninth day, after which porphyrin excretion rose rapidly. It is possible that saturation of the organism with the drug, or the precursor of the porphyrin, occurs during the initial period, since the phenomenon of delayed response was particularly striking in the case of substances like sulphanilamide and prosectasine which are poorly absorbed and less evident or absent when materials like aniline or hydroxylamine were dosed. It is also possible that a hepatotoxic effect might develop during the course of some days [compare Espersen, 1937].

At the end of an experiment, the animals were killed by coal gas and specimens of liver, spleen and kidney (sometimes also bone-marrow) taken in corrosive-acetic fixative for histological examination.

All preparations were examined for stainable iron (prussian blue reaction) in addition to haematoxylin-eosin differentiation.

#### *Isolation of the porphyrins*

The urine extracts from experiments with any one drug were pooled and the ether-soluble porphyrins isolated in the following manner.

The 0.5% HCl solution was filtered into a small separating funnel and covered with a layer of ether. A few drops of glacial acetic acid were then added and sufficient saturated aqueous potassium acetate to render the mixture neutral to Congo red. Upon shaking, the porphyrins passed up into the ether layer which was separated, washed well by shaking with water and allowed to evaporate to dryness. The residue was washed once with light petroleum and then esterified by dissolving in methyl alcoholic HCl (saturated at 0°). After 24 hr., chloroform was added and much water in a separating funnel. The chloroform layer, containing the porphyrin esters, was washed well with dilute  $\text{Na}_2\text{CO}_3$  and then water and evaporated to dryness. After rinsing with light petroleum, the dry residue was treated with cold, anhydrous ether until no more pigment passed into solution. This ether solution, containing the coproporphyrin III ester, was evaporated to a very small volume and set aside in a closed tube in the ice-box for crystallization. Any ether-insoluble pigment was dissolved in a little chloroform and to the concentrated solution about 5 vol. of hot methyl alcohol were added and the mixture set in the ice-chest. Any coproporphyrin I ester separating could be centrifuged down, but in many cases the quantity present was barely more than a trace, too small for identification by melting-point. The crystals of coproporphyrin III ester, which formed in the concentrated ether solution, were separated from mother liquor (removing the latter with a micro-pipette), washed sparingly with cold, anhydrous ether, then with light petroleum and dried over  $\text{P}_2\text{O}_5$  *in vacuo*. Melting-points were recorded on the Kofler electrically-heated micro-melting-point apparatus.

#### RESULTS

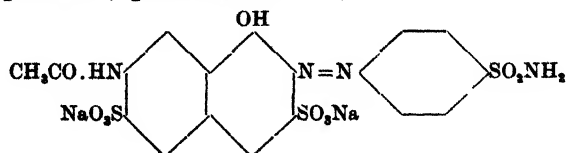
Economy of space prevents the full details being given for each substance tested. The data for experiments with sulphanilamide and aniline are given in full in the protocol in the Appendix; all the findings are presented in summary form in Tables I and II.

The following drugs were investigated:

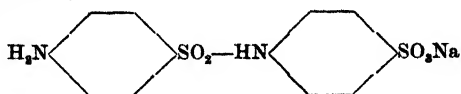
- (1) *p*-Aminobenzenesulphonamide ("sulphanilamide"):



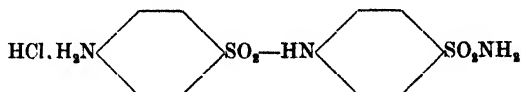
- (2) Disodium 4'-sulphonamidophenyl-2-azo-7-acetamino-1-hydroxynaphthalene-3:6-disulphonate ("prontosil soluble"):



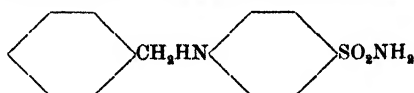
(3) Sodium *p*-aminobenzenesulphonyl-*p*'-aminobenzenesulphonate ("sodium sulphanilyl-sulphanilate"):



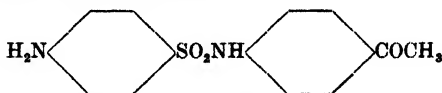
(4) *p*-Aminobenzenesulphonyl-*p*'-aminobenzenesulphonamide hydrochloride ("disulphanilamide hydrochloride"):



(5) *p*-Benzylaminobenzenesulphonamide ("proseptasine"):

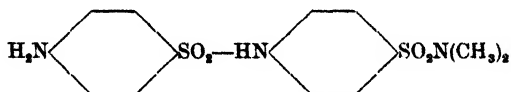


(6) 4-Sulphanilylamidoacetophenone:

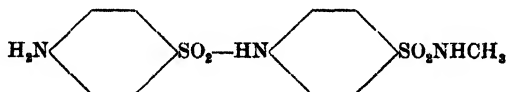


This substance was kindly placed at our disposal by the manufacturers, Messrs E. Merck, in consultation with the Therapeutic Trials Committee of the Medical Research Council. We are grateful to Messrs Merck and to the above-named Committee for permission to publish our findings.

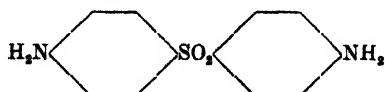
(7) *p*-Aminobenzenesulphonyl-*p*'-aminobenzenedimethylsulphonamide ("uliron"):



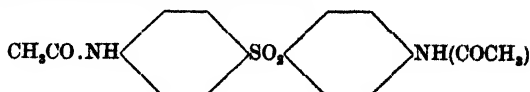
(8) *p*-Aminobenzenesulphonyl-*p*'-aminobenzenemethylsulphonamide ("disseptal B"):



(9) 4:4'-Diaminodiphenylsulphone:



(10) 4:4'-Diacetaminodiphenylsulphone:



(11) Sulphanilic acid.

(12) *p*-Aminobenzoic acid.

(13) *p*-Phenylenediamine.

- (14) Aniline, purified by redistillation.
- (15) Monomethylaniline (prepared by hydrolysis of the crystalline acetyl compound, "exalgin", and purified by redistillation).
- (16) Dimethylaniline (purified by repeated recrystallization and finally redistilled).
- (17) 2:4:6-Trichloroaniline.
- (18) Phenetidine (purified by redistillation).
- (19) Benzidine.
- (20) *o*-Toluidine.
- (21) 5-Chloro-*o*-toluidine.
- (22) Nitrobenzene (purified by redistillation).
- (23) Methylacetanilide ("exalgin").
- (24) Hydroxylamine hydrochloride.
- (25) Phenylhydroxylamine (freshly prepared by Imperial Chemical Industries and stored in N<sub>2</sub> in the dark).
- (26) Sodium nitrite.
- (27) *iso*Amylamine.

### DISCUSSION

A discussion of our results will be much facilitated by reference to the structural formulae on pp. 963-4 and to Tables I and II in which the main findings are summarized.

It will be seen that porphyrinuric activity is by no means limited to sulphanilamide, but is shared by most of the drugs belonging to the sulphonamide series. In addition, a number of the simple aromatic amines tested, particularly aniline and its derivatives, were found to possess this property to a marked degree.

No doubt, were it possible to give sufficiently high doses, it would be found that a great number of substances would cause porphyrinuria, but on the basis of the dosage rates employed in this investigation it would seem permissible to draw certain general conclusions, relating porphyrinuric action and therapeutic efficiency to chemical constitution, as follows.

(1) *There is no quantitative relation between therapeutic efficiency and porphyrinuric action.* Within the series of sulphonamide drugs, it is not always easy to compare therapeutic action with toxicity on account of the fact that many of the substances are sparingly soluble and poorly absorbed so that only a small proportion of the ingested dose actually finds its way into the blood stream. Nevertheless, substances like sulphanilamide and disulphanilamide, for example, are considered to exert comparable therapeutic effects and yet the latter drug does not cause any increase in porphyrin excretion in the dosage attainable. Similarly, many of the simpler substances related to the sulphonamides have been shown to increase very markedly the urinary porphyrin excretion and yet they are devoid of therapeutic action in experimental infections.

(2) *In general, porphyrinuric action runs roughly parallel with general toxicity.* An apparent exception to this generalization is the case of 4:4'-diacetamidodiphenylsulphone, which is tolerated in single doses > 40 g./kg. but which produces quite a pronounced porphyrinuria when administered daily at the level of 0.18 g./kg. These amines and their acetyl derivatives are known to come into a state of dynamic equilibrium *in vivo* and hence one might expect the toxicity of the diacetyl derivative of the sulphone to be much greater (approaching that of the parent amine) when given regularly than when administered on a single occasion [compare Nitti *et al.* 1938]. The post-mortem histological changes suggest a similar action. Moreover, it must again be pointed out that the

Table I

No.	Drug	Porphyrinuric action					M.P. and re-m.p. of Me ester of porphyrin isolated	Methb formation	P.M. histological findings	Remarks
		M.Pro.D.* g./kg. 0.25-1	M.L.D.† g./kg. 4	Dose g./kg.	Porphyrin excretion	( × normal)				
1	Sulphanilamide			0.4	+	2.5	142-4°,	+	Liver, small amount of stainable iron and some brownish pigment. Kidney, some brownish pigment in convoluted tubules. Spleen, intensely engorged, much stainable iron, also brownish pigment	There was a marked increase in water intake and in urine vol. accompanied by loss in body wt.
				0.93	+	4	160°			
				1.23	+	5				
				1.39	++	7				
				1.42	++	6.5				
2	Prontosil soluble	1	4 (i.m.†)	1.25 (i.m.)	+	6	146°,	+	Spleens of groups on larger dose were engorged, contained brown amorphous pigment and stainable iron. Remaining organs and second group normal	
				2.10	++	13	168°			
3	Na sulphanilyl-sulphanilate	s.c.§ effective; per os 2	20	0.05	-			-		Slight loss in body wt. Urine vol. unchanged. Slight local reaction at site of s.c. injection  Animals gained in wt. High M.L.D. and absence of porphyriuric action, probably due to poor absorption with resulting low concentration in blood [Feinstone <i>et al.</i> 1938]
4	Disulphanilamide hydrochloride	0.25-1	20	0.25	-			-		
				1.0 (s.c.)	-			-		
				0.53	-			-		
				0.80	-			-		

5	Proseptasine	0.5	20	1.0	+	2	Slight engorgement of spleen with some deposits of stainable iron
6	4-Sulphanilyl-amidoaceto-phenone	0.5	>10	0.55 1.15	++ ++	7.6 7.1	Much brown pigment and stainable iron in spleen
7	Ulliron			1.0	±	-	Loss of body wt. Urine vol. unaltered
8	Disceptal B			1.0	+	3	Marked loss in wt. and moderate increase in urine vol. Coats became rough and staring Wt. and urine vol. unchanged. Ulliron has caused fatal funicular degeneration with an inflammatory reaction of the spinal cord [Schubert, 1938]. Both nos. 7 and 8 cause polyneuritis [Hüllstrung & Krause, 1938, 1, 2] but cyanosis only infrequently
9	4:4'-Diaminodiphenylsulphone	0.0025 -0.01	0.25-0.5 <sup>†</sup>	0.028 0.045 0.080 0.15	++ ++ + Hburia + (deaths)	3.5 3.5	Small deposits of stainable iron in the spleen, but in general much less marked than in the case of sulphanilamide
10	4:4'-Diacetaminodiphenylsulphone	0.25-0.1	>40	0.18 0.59	++	4 5	Marked loss in body wt. Hyperexcitability and loss of hair. Cyanosis very marked

\* M.Pro.D. = Minimal protective dose, mouse or rat.

† i.m. = Intramuscularly.

§ s.c. = Subcutaneously.

† M.L.D. = Minimal lethal dose, mouse or rat.

‡ Repeated doses.



Table II

Porphyrinuric action					P.M. histological findings	Remarks
No.	Substance	Dose g./kg.	Porphyrin excretion	( $\times$ normal)	Me ester of porphyrin isolated	
11	Sulphanilic acid	1.4 1.8	- -			Smaller dose administered as Na salt, dissolved in drinking water. Lack of effect and of therapeutic activity may be due to high solubility or to dipole nature of the molecule Substance also a dipole
12	<i>p</i> -Aminobenzoic acid	1.25	-			
13	<i>p</i> -Phenylene-diamine	0.18 0.27	$\pm$ $\pm$			
14	Aniline	0.35 0.59	+ ++	3 12	139°	Causes dermatitis. The urine became very dark in colour Well-known cause of methaemoglobinæmia and of bladder tumours. Porphyrinuric action not previously discovered. Urine vol. increased by about 60%, very dark in colour but Hb absent. Nicotinic acid (0.1 g. per rat per day) had no effect on the porphyrinuria
15	Monomethyl-aniline	0.025 0.125	+ ++	3 5.5	133-7°	
16	Dimethylaniline	0.064 0.26	$\pm$ +	1.6 1.8	158°	Dimethylaniline can be demethylated <i>in vivo</i> by the dog, cat and rabbit [Horn, 1936, 1, 2], the latter, however, only removing one Me group
17	2,4,6-Trichloro-aniline	0.01 0.054 0.625	- $\pm$ +			Substance dosed dissolved in olive oil. Both <i>o</i> - and <i>p</i> -positions are occupied thereby, preventing oxidation <i>in vivo</i> to an <i>o</i> - or <i>p</i> -iminoquinone
18	Phenetidine	0.044 0.22	+ +	2 3.5	137°	Engorgement of spleen but no increase in stainable iron

19 Benzidine	0.25	+	+	7.5	153°	+	Engorgement of spleen with fairly abundant deposits of stainable iron. Some also present in liver	Well known to be a cause of cyanosis and intoxication among industrial workers, who inhale the light powdered substance
20 <i>o</i> -Toluidine	0.11	+	+	3.5		+	Slight deposition of stainable iron in spleen	
21 5-Chloro- <i>o</i> -toluidine	0.15	+	+	3.5		+	Some vacuolation of liver cells. Spleen engorged and contained much stainable iron	Substance dosed dissolved in olive oil. Causes both cyanosis and dermatitis in man. All animals lost wt.
22 Nitrobenzene	0.125	+	+	2	146°, 170°	+		Well known to cause cyanosis and "anilism" among industrial workers. The nitro compounds can be reduced <i>in vivo</i> to the corresponding hydroxylamines or amines
23 Methylacetanilide	0.11 0.13	-	-			-		Animals exhibited nervous excitability and twitching. The drug differs from acetanilide in that having the amino group completely substituted, it does not cause methaemoglobinæmia
24 Hydroxylamine-hydrochloride	0.075 0.15	+	+	4.5	145°, 170-1°	+	Liver and kidneys contained brownish black particles in which the iron stain was positive. Spleens engorged, but otherwise normal	The substance is an active haemolytic agent [de Langen & Grotepass, 1938]
25 Phenylhydroxylamine	0.014	+	+	3.3		+		Animals markedly cyanosed. This substance or a derivative is postulated as an intermediate in the oxidation of all the amines here investigated (see Discussion)
26 Na nitrite	0.18	-	-			+	Liver showed engorgement with vascular dilatation. Otherwise organs normal	Administered 10 mg. <i>per os</i> daily and a 0.4% solution in the drinking water. Porphyrin excretion decreased to nearly nil. Urine colour normal. Possibly some degree of anaemia
27 <i>iso</i> -Amylamine	0.11	-	-			-		

figures quoted by various workers for the minimal fatal dose of these sparingly soluble compounds are apt to be misleading. The quantity of drug reported as being tolerated in a single dose may sound impressive, but in all probability the greater part of the material is not absorbed.

(3) *In general, all those substances found to exert a porphyrinuric action are also capable of producing methaemoglobinaemia in vivo and*

(4) *one can therefore define the chemical grouping necessary for increasing porphyrin excretion in the same terms as allotted to the structure giving rise to methaemoglobin formation—viz. the presence of an aromatic amino group, unsubstituted or potentially free and preferably in such a system that it is capable of undergoing oxidation with the formation of a hydroxylamine derivative or a reversibly oxidizing system such as a p- or o-iminoquinone.*

The role of the primary amino group in sulphanilamide in determining the toxicity of this substance has already been suspected [Jennings & Southwell-Sander, 1937], largely as a result of the work of Kracke & Parker [1933-4].

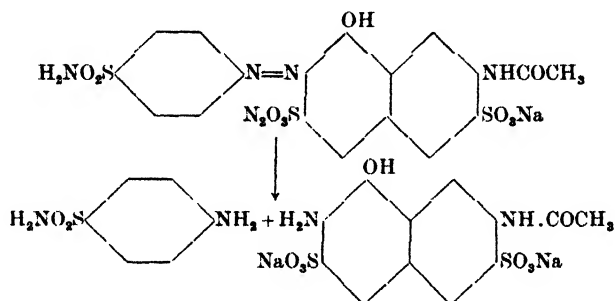
More recently, a striking occurrence reported by Rogers [1938] would appear to support the idea that the amino group is the structural feature, possessed by the sulphonamide drugs in common with certain other substances, which is largely responsible for their specific toxic effects. A woman of 58 was receiving sulphanilamide treatment for erysipelas. Some years previously the local anaesthetic "procain" (*p*-aminobenzoyldiethylaminoethanol hydrochloride) had been administered with evident indications of sensitization. After ingestion of as little as 1.6 g. of sulphanilamide, reactions occurred at all the old sites of injection in a roughly quantitative manner. Patch tests were performed and proved consistently positive to procain but not to sulphanilamide. The author, however, concluded that the feature which these chemical substances exhibited in common was the possession of an aromatic primary amino group. It appears to the writers unfortunate that patch tests were not tried also with phenylhydroxylamine and *p*-aminophenol.

It will be seen, when conclusion no. 4 is examined more carefully with reference to the summarized results in Table I, that not only is there a qualitative correspondence between methaemoglobin production and porphyrinuric action, but that the relationship is also roughly quantitative. Thus aniline, *o*-toluidine and benzidine are well known by experience to cause severe methaemoglobinaemia and they also cause a prompt rise in porphyrin excretion at a relatively low level of dosage. Dimethylaniline, although fully substituted, yet causes a mild degree of methaemoglobinaemia [compare Horn, 1936, 2], and even most carefully purified material was also found to be effective in causing a relatively weak porphyrinuria. *p*-Phenylenediamine, in spite of its tendency to cause dermatitis etc., induces neither methaemoglobin formation nor porphyrinuria. The drug "exalgin" (methylacetanilide) is similarly ineffective, and 2:4:6-trichloroaniline, in which both *p*- and *o*-positions are substituted, is practically ineffective in both respects. The finding with nitrobenzene must also be mentioned. The nitro-compounds are known easily to suffer reduction in the animal body and give rise to methaemoglobinaemia just as do the corresponding amines; in fact the nitro-compounds are perhaps somewhat more dangerous. In the animal experiment it was found by us that so low a dose of nitrobenzene as 0.125 g./kg. brought about a distinct and prompt porphyrinuria. It will be noticed that prontosil is relatively more effective in causing increased porphyrin excretion than is sulphanilamide, when the doses are compared upon the basis of potential equivalence in sulphanilamide content. Prontosil is known to be

transformed, in part at least, into sulphanilamide in the animal body [Fuller, 1937; Jauerneck & Gueffroy, 1937] and could theoretically yield 29.27% of its weight of sulphanilamide.

	Dosage g./kg.	Sulphanilamide equiv. g./kg.	Increase in porphyrin times normal
Prontosil soluble	1.25	0.366	6
	2.1	0.62	13
Sulphanilamide	0.4	0.4	2.5
	0.93	0.93	4

The transformation in the living organism would involve a reduction of the azo linkage giving, in addition, another amino-compound thus:

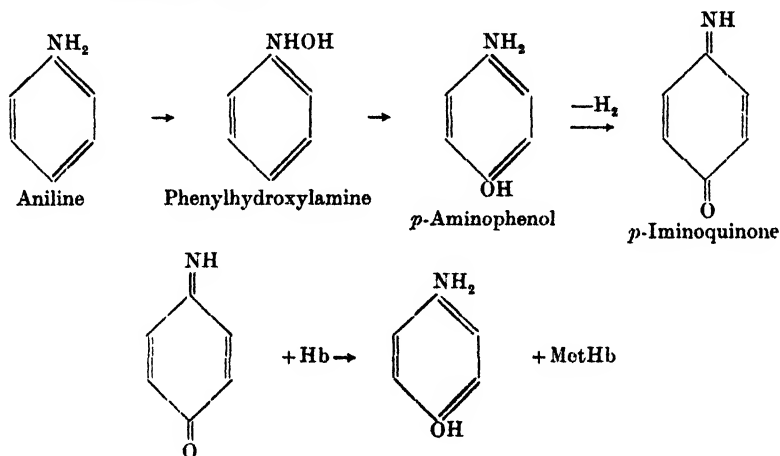


Since one of the conclusions reached as a result of the present investigation is that aromatic amino compounds are potentially capable of producing increased porphyrin excretion, the greater magnitude of the effect produced by prontosil soluble may be due to the fact that both the sulphanilamide and the naphthylamine derivative produced during its biological fission contribute towards porphyrin excretion and the net result is therefore additive. Attention, however, must also be drawn to the fact that the prontosil was given intramuscularly and the sulphanilamide by mouth.

The discovery of this relationship between methaemoglobin-forming properties and porphyrinuric action would seem to be of considerable significance. Not only does it define the chemical structure in the drug responsible for the effect and thereby throw open the possibility of synthesizing a compound free from this adverse characteristic, but possibly still capable of exerting the required chemotherapeutic effects (attempts in this direction have already been made by Michaux *et al.* [1938] who prepared the Na methylenesulphonate derivative of sulphanilamide, and by McLeod [1938]), but it also serves to associate these two manifestations of the toxic action of the sulphonamide drugs upon the haemopoietic system.

Primary aromatic amines, e.g. aniline, can undergo oxidation in the animal body with the formation of phenolic derivatives such as *p*-aminophenol, a reaction in which the hydroxylamine derivative is probably an intermediate. Thus Young & Wilson [1926] were able to detect *p*-aminophenol in the blood plasma and urine after administration of acetanilide to rabbits, whilst Ellinger [1920] recovered acetylphenylhydroxylamine from the blood of acetanilide-poisoned cats, and Lipschitz [1920] has demonstrated the reduction of *m*-dinitrobenzene to *m*-nitrophenylhydroxylamine by minced animal tissues *in vitro*. Heubner, however, does not consider the hydroxylamine to be the actual methaemoglobin-former, since it is irreversibly oxidized *in vivo* with ease to

azoxybenzene [Heubner *et al.* 1923], and his own studies have shown, in agreement with the work of others, that aniline introduced into the blood stream may bring about the formation of many times its equivalent of methaemoglobin; in fact it appears as if an equilibrium is established when approximately one-third of the total blood pigment has been oxidized. Aminophenol, on the other hand, is known to pass very readily and reversibly into *p*-iminoquinone. This could oxidize one equivalent of haemoglobin into methaemoglobin, becoming reduced in the process, be oxidized again by the tissues and enter into reaction with a further molecule of haemoglobin. It would thus act as a catalyst, converting more and more of the blood pigment into methaemoglobin until an equilibrium was eventually established, or it itself became destroyed. The scheme is illustrated by the following diagram:



Should a similar mechanism be entertained as responsible also for methaemoglobin formation in the case of sulphanilamide and its homologues (as first suggested by Rimington [1939]), then evidence must be adduced that the primary aromatic amino group of these compounds is susceptible to such an *in vivo* oxidation as that postulated. Ottenberg & Fox [1938] have shown that on irradiating dilute aqueous solutions of sulphanilamide with ultra-violet light, a rapid change takes place, the solution becoming first purple-coloured and then yellow-brown. Simultaneously there is a decrease of 30–60% in diazotizable amine, determined by Marshall's method. Ottenberg and Fox did not determine the type of change affecting the amino group (see also Main *et al.* [1938]), but repetition of their experiments has convinced us that oxidation plays a prominent part since (1) no colour change takes place when all air is replaced by nitrogen; (2) aeration during the process of irradiation greatly accelerates and intensifies the reaction; (3) the purple-coloured product is reversibly decolorized by a reducing agent such as sodium hydrosulphite (the yellow-brown is not and would appear to represent a further stage of greater complexity, possibly analogous to the transformation of aniline into aniline black); and lastly, (4) methylene blue may replace oxygen in the system as a hydrogen acceptor. Irradiation of sulphanilamide solution under nitrogen and in presence of dilute methylene blue leads to decoloration of the latter.

From these observations we feel justified in regarding the irradiation phenomenon as one of oxidation in which the amino group is involved.



The conclusion that oxidation of a primary amino group is responsible for the porphyrinuric action of the sulphonamide drugs, etc., is strongly supported by the finding of Brownlee (personal communication of unpublished results) that a similar increase in urinary porphyrin excretion is caused by another class of pharmaceuticals, namely the antipyretic drugs, phenacetin, phenazone etc., which contain a free or substituted aromatic amino group.

Important though our conclusions concerning the phenomena of methaemoglobin formation and porphyrinuric action appear to us to be for a general understanding of the toxicity of the sulphonamide drugs and their action upon the haemopoietic system, yet we feel that our work would not be complete without some discussion of the possible origin of the extra porphyrin excreted.

Obviously the question which first presents itself is whether excreted coproporphyrin is actually derived from the methaemoglobin of the blood stream. In all instances, the porphyrins isolated after the administration of the drugs we have employed, belonged to the isomeric series III, so that such an origin would be chemically possible. It has, however, become customary to accept the view of haemoglobin degradation put forward by Lemberg [1935] in which the haematin moiety of haemoglobin is pictured as suffering oxidative ring scission whilst the iron and globin are still attached to the molecule and so passing to biliverdin without at any time going through a porphyrin stage. Porphyrins are thus unlikely to arise from the normal breakdown of haemoglobin and there is much physiological evidence to support the correctness of this viewpoint. Recently, however, we have been studying the fate of methaemalbumin formed by injecting haematin into the blood stream of rabbits, man or monkeys and we have evidence that this pigment ultimately appears, in part at least, as extra proto- and copro-porphyrin in the faeces. That haematin injection does not lead to increased bile pigment formation, as does the injection of haemoglobin, has been known for some time [cf. Duesberg, 1933-4]. An increased excretion of coproporphyrin III has also been observed by several workers to occur in certain types of hepatic disease, and in many of these, as reported by Schumm [1927], haematinaemia has been demonstrated.

It would appear, therefore, that not only does there exist the haemoglobin-verdohaemochromogen-biliverdin mechanism of blood pigment degradation, but that, in addition, an alternative route exists by which haematin, the oxidized haem component detached from globin, is metabolized in pathological conditions, at least, but perhaps also normally [compare Thomas, 1938]. It is conceivable that methaemoglobin, if formed in quantity, might follow the same route. Heubner considers that the rate of reconversion of methaemoglobin into haemoglobin *in vivo* is not so great as the earlier experiments of Sakurai [1925] would lead one to suppose.

From histological examination of the organs, and particularly the spleens, of the rats made porphyrinuric by the dosing of sulphanilamide, aniline etc., it is highly probable that an increased red cell destruction has been caused by these drugs. The spleens exhibit a considerable degree of haemosiderosis with dilatation and engorgement [compare Hageman, 1937; Schwartz *et al.* 1938, etc.]. Machella & Higgins [1939] have recently demonstrated the production of severe anaemia by the dosing of sulphanilamide to rats.

Tentatively, therefore, we put forward the view that these drugs, capable of suffering oxidation at the amino group *in vivo*, by causing methaemoglobinaemia and increased disintegration of erythrocytes, bring about an increased excretion of type III porphyrins derived from the broken down blood pigment. What role, if any, impairment of normal liver function may play in this sequence of events

we are at present not able to state with certainty. A severe hepatotoxic action of sulphanilamide has been reported in cases by Garvin [1938] and by Cline [1938].

#### SUMMARY

The finding, already published, that sulphanilamide, when dosed to rats, causes an increase in the urinary and faecal porphyrin excretion, has been confirmed and amplified by extending the investigation to include other drugs of the sulphonamide series and also simpler related chemical substances. From the results which are now reported in detail, with chemical and pathological findings, the following general conclusions may be drawn.

(1) Within the series of sulphonamide drugs tested, there is no quantitative relation between therapeutic efficiency and porphyrinuric action.

(2) Porphyrinuric action runs roughly parallel with general toxicity.

(3) In general, all those substances found to exert a porphyrinuric action are also capable of producing methaemoglobinaemia *in vivo*.

(4) One can therefore define the chemical grouping necessary for increasing porphyrin excretion in the same terms as allotted to the structure giving rise to methaemoglobin formation—viz. *the presence of an aromatic amino group, unsubstituted or potentially free and preferably situated in such a system that it is capable of undergoing oxidation with the formation of a hydroxylamine derivative or a reversibly oxidizing system such as a p- or o-iminoquinone.*

The last generalization has been discussed in the light of Heubner's work upon the mechanism of methaemoglobin formation following administration of aniline. Both aniline and nitrobenzene (which can suffer reduction *in vivo*) have been shown to be very effective porphyrinuric agents and attention has been drawn to the possible importance of this finding in connexion with the control of the health of industrial workers.

The evidence has been considered which supports the hypothesis that sulphanilamide, etc., may to some extent undergo oxidation at the amino group *in vivo*.

The source of the extra coproporphyrin III excreted as a result of the administration of these substances, has also been considered, and the hypothesis is put forward that it is derived from an increased breakdown of blood pigment. It is suggested that methaemoglobin, when once formed, is degraded, in part at least, by a mechanism which leads ultimately not to bile pigment but to porphyrin. Haematin may possibly represent one of the intermediary stages in this transformation. Further work is in progress designed to elucidate the exact nature of the changes involved.

We wish to thank Dr Buttle, of the Wellcome Physiological Research Laboratories, for generous gifts of some of the drugs and for his kindness in supplying information concerning the effective and fatal doses of some of the compounds investigated. We are also indebted to Dr M. W. Goldblatt and Imperial Chemical Industries for kindly supplying some of the substances used in this investigation, and to Bayer Products, Ltd., for the Prontosil, Uliron and Disseptal B.



## APPENDIX

*Protocol of experiment with sulphanilamide*

Three male rats, av. wt. approximately 200 g., in each group. Groups 36 and 37 and control gained in wt. during the exp., whilst group 38 lost in wt.

	Group 38 (0.93 g./kg.)		Group 36 (1.23 g./kg.)		Group 37 (1.42 g./kg.)		Controls	
	Porph. μg.	Vol. ml.	Porph. μg.	Vol. ml.	Porph. μg.	Vol. ml.	Porph. μg.	Vol. ml.
Pre-period								
i-iii	9.8	40	Trace	50	15.3	52	20.7	23
iv-vi	11.2	62	6	62	5.0	69	30.2	34
vii-ix	10.8	61	13.5	63	10.8	88	29.9	37
x-xii	14.1	66	11.4	53	12.8	66	32.9	31
Days on drug								
1-3	19.1	166	27.2	186	29.9	175	32.7	25
4-6	15.9	97	30.5	182	29.9	131	23.3	27
7-9	46.8	81	35.6	120	40.8	105	30.2	24
10-12	38.6	97	44.0	169	29.1	198	38.7	29
13-15	53.6	82	63.4	169	68.0	124	31.1	29
16-18	45.0	74	64.9	169	62.6	121	—	—

*Protocol of experiment with aniline*

The average body wt. decreased slightly during the exp.

Group 13. Aniline 0.59 g./kg. per day.

	Urinary porphyrin in μg.		Urine vol. in ml. Group 13
	Group 13 3 young ♂	Controls 3 ♀	
Pre-period (mean)	9	10	73
Days on drug 1-3	110	6	106
4-6	110	7.5	120
7-9	110	7.5	103
10-12	110	7.5	137
13-15	130	7.5	105

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# CXIX. INTERMEDIARY CARBOHYDRATE METABOLISM IN AMPHIBIA

## I. CARBOHYDRATE BREAKDOWN BEFORE METAMORPHOSIS

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*(Received 1 April 1939)*

CARBOHYDRATE metabolism seems to be of dual importance in embryonic life. In the earlier stages of development it is believed that the active substance which brings about neural induction (the evocator) forms a symplex with glycogen and protein and when this is broken down in the dorsal lip of the blastopore, the evocator is liberated [Waddington *et al.* 1935; Waddington *et al.* 1936; Needham, 1936]. In older stages the breakdown of carbohydrate probably provides much of the energy necessary for growth and differentiation.

An extensive study of carbohydrate metabolism in the chick embryo [Needham & Nowiński, 1937; Needham *et al.* 1937; Needham & Lehmann, 1937, 1, 2; Lehmann & Needham, 1937; 1938] showed that the way of carbohydrate breakdown in chick embryo probably differs from that in muscle. The formation of lactic acid in the embryo seems not to pass through a phosphorylating cycle.

The main characteristics of embryonic carbohydrate metabolism found by these authors may be summed up as follows: glycogen is not broken down to lactic acid, whereas glucose and mannose are readily used. The intermediate products of phosphorylation, such as hexose-monophosphate or -diphosphate are not used at all and the same applies to other carbohydrates such as fructose, galactose, sorbose, various pentoses, glucosamine etc. Embryo glycolysis somewhat resembles glucolysis in brain and in tumour tissue. It may be inhibited by *dl*-glyceraldehyde [Needham & Nowiński, 1937] as Mendel [1929] found for tumour tissue. Fluoride also inhibits glucolysis in embryo, but there is no accumulation of hexosediphosphate or phosphoglyceric and glycerophosphoric acids [Needham *et al.* 1937].

These observations on chick embryo seemed sufficiently interesting to warrant the question whether they are typical for embryonic tissue in general. In the present work the carbohydrate breakdown in tadpole tissues (*Rana temporaria*) before metamorphosis has been studied. A later paper will deal with the carbohydrate metabolism during metamorphosis. Studies on mammalian embryo are also planned.

### *Methods*

The methods described previously [Needham & Nowiński, 1937; Needham *et al.* 1937] were used in the present work. Substrate preference and glyceraldehyde inhibition were studied manometrically but in smaller Warburg cups (6.0–7.0 ml.). The temp. of the water bath was 20°. The first reading was taken 20–30 min. after gassing with N<sub>2</sub>/CO<sub>2</sub> mixture.

As the younger tadpoles after hatching still contain much yolk and the tissues are saturated with their natural substrate, no significant results could be expected from experiments on this stage. Work was begun, therefore, with stages in which no trace of yolk in the tadpole could be seen under the binocular microscope.

The absolute age of the animals was hard to determine as the eggs were sometimes found already fertilized in the laboratory tanks, but the youngest stages investigated were about 5 days after hatching. The size of the tadpole, as a criterion of age, is not very dependable, but it led to the interesting result that the chemical differentiation (of enzyme systems) of the tissues seems to be independent of growth in length.

If brei is used, the tadpoles being ground in a small mortar until morphological structure is destroyed, the  $Q_L^N$  obtained is so low (for autoglycolysis +1.65, for glucose as substrate +2.85) that the enzyme system is obviously inactivated by the process. Experiments on intact tadpoles were also not very successful as the  $Q_L^N$  for autoglycolysis and glucose as substrate were almost the same (autoglycolysis +12.7 and glucose +13.95). Probably the cutis is not permeable to these substances.

The embryos were therefore pulped with a scalpel, but not into too small pieces. The  $Q_L^N$  thus obtained were more satisfactory (e.g. in Exp. 5 for 10–12 mm. tadpoles: autoglycolysis +10.48; with glucose as substrate +19.2).

The appropriate carbohydrates were added before the experiments started. As the added substances were dissolved in  $\text{NaHCO}_3$ -Ringer for the frog, no dilution occurred of Ringer solution present in the cup.

#### *Autoglycolysis*

Table I shows the autoglycolysis of tadpoles during development. In these experiments the total length of the tadpoles was taken as the criterion of age (the figures were mainly obtained from the same lot of tadpoles, measured as development progressed).

Table I

Size of tadpoles in mm.	Average $Q_L^N$
8–10	+ 19.35
10–12	+ 12.7
12–14	+ 12.1
16–18	+ 10.72
18–20	+ 11.9
20–22	+ 14.6

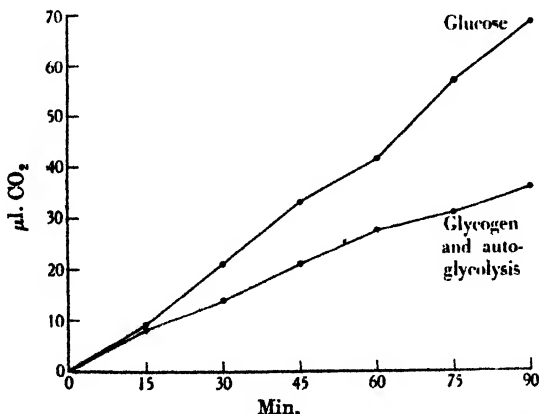


Fig. 1. Preference for glucose. A record of a single experiment.

The curve of autoglycolysis is quite similar to the curve of autoglycolysis in chick embryo [Needham & Nowiński, 1937]: it decreases rapidly with age (Fig. 1). The unusually high  $Q_L^{N^*}$  of +14.6 for the 20–22 mm. tadpoles is probably due to "cannibalism": the tadpoles in my experiments were given no food, but in some cases it was impossible to prevent them from eating weak or dying individuals. Fed animals of course grow more quickly but though the size is greater (e.g. 20–22 mm.) the chemical differentiation is just the same as that of 10–12 mm. tadpoles, judged by substrate preference.

#### *Substrate preference*

As in the case of chick embryo, glucose but not glycogen is broken down by tadpole tissue. Fig. 1, a record of a single experiment, shows that the curve of glycogen breakdown (in ml.  $\text{CO}_2$ ) runs closely with the curve of autoglycolysis (in all the cups there was approximately the same amount of tissue and the vessel constants differed only slightly), whereas the glucose breakdown gives much higher values, and at the end of the exp., when the glycogen breakdown reaches the value of  $36\mu\text{l. CO}_2$ , the  $\text{CO}_2$  production with glucose as substrate rises to  $67.5\mu\text{l.}$  The corresponding values for  $Q_L^{N^*}$  in this experiment were: autoglycolysis +11.45, glycogen +11.9, glucose +22.5.

These facts were quite reproducible and the main results are collected in Table II. Though there is some scatter among the various experiments, nevertheless the preference for glucose during the course of development of the tadpole is quite obvious.

Table II

Length of tadpoles in mm.	$Q_L^{N^*}$ glycogen	$Q_L^{N^*}$ glucose
8–10	+19.0	+27.0
10–12	+10.48	+19.2
—	+13.5	+22.25
—	+8.5	+29.0
—	+15.2	+27.3
12–14	+16.1	—
14–16	+19.2	+30.3
—	+12.0	+21.0
—	+11.26	—
—	+7.64	+17.51
16–18	+12.6	—
20–22	+20.25	+25.0

Table III

Exp. no.		$Q_L^{N^*}$
8	Autoglycolysis	+13.5
	Hexosediphosphate	+12.0
	Glucose	+29.0
9	Autoglycolysis	+10.83
	Hexosediphosphate	+10.3
		+10.67
10	Autoglycolysis	+11.5
	Hexosediphosphate	+11.8
		+10.55
		+11.08

Table III shows that adding hexosediphosphate has no effect upon glycolysis, the  $Q_L^{N^*}$  for hexosediphosphate corresponding closely to the  $Q_L^{N^*}$  of autoglycolysis, whereas glucose (as in Exp. 8) gives a value 70 % higher.

From these results we may draw the conclusion that the mechanism of glucolysis in the tadpole is similar to the glucolysis of the chick embryo.

### *Glyceraldehyde inhibition*

It was first observed by Mendel [1929] that *dl*-glyceraldehyde has the property of inhibiting glucolysis of tumour tissue completely. The same effect may also be produced on the glucolysis of brain [Ashford, 1933], but not on the glycolysis in muscle extract as mentioned by Holmes [1934] and confirmed by other investigators [Needham & Lehmann, 1937, 2; Baker, 1938; Cori *et al.* 1939]. It was therefore suggested that *dl*-glyceraldehyde is a specific inhibitor of glucolysis. As such it was used by Needham and co-workers [Needham & Nowinski, 1937; Needham & Lehmann, 1937, 2] for embryonic tissue and it was found that in chicken embryo glyceraldehyde inhibits the breakdown of glucose. This effect is due to *l*-glyceraldehyde only as was shown by Needham & Lehmann [1937, 2] and confirmed for tumour tissue by Mendel *et al.* [1938]. The specificity of this inhibition was contradicted by Boyland & Boyland [1938] and by Adler *et al.* [1937]. The latter authors claimed to show that glyceraldehyde inhibits not only glucolysis in tumour tissue but also the glycogen breakdown in muscle extract. This discrepancy between the results of these authors and those of Holmes, Needham & Lehmann, Baker and Cori *et al.* was removed by Lehmann & Needham [1938] who showed that freshly prepared glyceraldehyde (in dimeric form) inhibits both ways of carbohydrate breakdown, glucolysis and glycolysis, whereas glyceraldehyde in its monomeric form (produced by standing for some time in solution) acts as a specific inhibitor of glucolysis.

It was of interest to investigate whether tadpole tissue behaves towards this specific inhibitor of glucolysis in the same way as tumour, brain or chick embryo. The results obtained in these experiments showed that it does. Of course stronger concentrations ( $5 \times 10^{-2} M$ ) inhibit glucolysis: in Exp. 25 for instance  $Q_L^N$  for glucose as substrate was +19.8 but for glucose + glyceraldehyde +8.77 only. In another exp., where  $Q_L^N$  for autoglycolysis was +4.55 and for glucose +11.59, the  $Q_L^N$  for glucose + glyceraldehyde was +3.58.

On the other hand Lehmann & Needham [1938] emphasize the importance of a suitable concentration: too strong concentrations may inhibit glycolysis and glucolysis [confirmed by Süllmann, 1939] as in the experiments of Boyland & Boyland and Adler *et al.* Lehmann & Needham obtained maximum inhibition with a glyceraldehyde concentration of  $3-6.7 \times 10^{-2} M$ . It was therefore necessary to test lower concentrations than stated above.

The experiments were carried out in Warburg manometers. Glucose and glyceraldehyde were added together and another manometer with glucose was used as a control. A further control consisted of tissue without substrate.

The rate of inhibition obtained from these experiments is shown in Fig. 2, being an average from various figures. It is obvious from it that with increasing concentrations the rate of inhibition rises. A glyceraldehyde concentration of

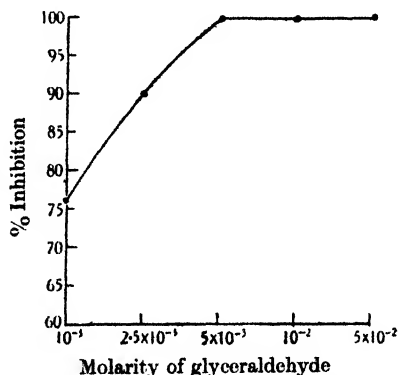


Fig. 2. Inhibition of glucolysis by glyceraldehyde.

$10^{-3} M$  inhibits 76 % of glucolysis;  $2.5 \times 10^{-3} M$  inhibits about 90 %; whereas  $5 \times 10^{-3} M$  inhibits glucolysis completely. The corresponding figures for  $Q_L^{N_2}$  are shown in Table IV.

Table IV

Concentration of glycerald.	$Q_L^{N_2}$ glucose + glycerald.	$Q_L^{N_2}$ glucose	$Q_L^{N_2}$ no substrate
$10^{-3} M$	17.75	34.00	12.65
$2.5 \times 10^{-3} M$	10.21	21.15	8.77
$5 \times 10^{-3} M$	6.55	12.30	8.28
$10^{-3} M$	1.70	12.30	8.28
$5 \times 10^{-3} M$	8.79	18.00	8.73

Lehmann & Needham [1938] stated that maximum inhibition has to be produced by a concentration of  $3 \times 10^{-3} M$  glyceraldehyde, but not stronger. My results are in accordance with this criterion as the maximum inhibition in my case lay between  $5 \times 10^{-3} M$  and  $2.5 \times 10^{-3} M$ . We may, therefore, draw the conclusion that glucolysis in the tadpole resembles that of chick embryo, tumour and brain tissue, in that it may be inhibited by glyceraldehyde.

#### *Triosephosphate accumulation*

Needham & Lehmann [1937, 1] showed that in the case of the chick embryo triosephosphate accumulates during the experiment. The following typical experiment shows the method.

*Exp. 20.* One hundred tadpoles (length 12–15 mm.) were chopped and incubated anaerobically for 1 hr. in Thunberg tubes (some experiments were carried out in Warburg cups). The first tube (X 31) contained tissue without substrate, tube X 52 an appropriate amount of hexosediphosphate and tube X 60 glucose. The amount of tissue was exactly the same in each tube: 2 ml. of brei. After 1 hr. incubation (anaerobically) at  $20^\circ$  the contents of these tubes were ground ice-cold with P-free sand and 3 ml. 3 % trichloroacetic acid, and filtered through asbestos in a Gooch crucible. 1 ml. of the filtrate was removed for estimation of the inorganic P. 1 ml. of filtrate was made alkaline (2N NaOH) and after standing for 20 min. at room temp., the P estimation (Fiske-Subbarow) was carried out. The difference of the two estimations gave the amount of triosephosphate.

The results of my experiments are shown in Table V. The differences between the alkali-labile + inorganic P and the inorganic P are very small. They are within the range of colorimetric experimental error. It would therefore appear that hexosediphosphate is not attacked at all by tadpole tissue.

Table V

No. of cup	Substrate	Inorganic P (mg.)	Alkali-labile P + inorganic P (mg.)
X 31	None	0.075	0.055
14	None	0.079	0.080
11a	None	0.039	—
X 52	Hexosediphosphate	0.051	0.069
11	Hexosediphosphate	0.054	0.073
13	Hexosediphosphate	0.049	0.058
X 60	Glucose	0.054	0.062
13	Glucose	0.071	0.079
14	Glucose	0.062	0.045

## SUMMARY

1. The anaerobic glycolysis of tissues of tadpoles between loss of yolk and metamorphosis was investigated.
2. As in the case of the chick embryo, tadpole autoglycolysis decreases in the course of development.
3. Tadpole tissue shows a preference for glucose; glycogen and hexosediphosphate are not broken down.
4. *dl*-Glyceraldehyde in a concentration  $5 \times 10^{-3} M$  inhibits by about 90 % the glycolysis of tadpole tissue.
5. These facts suggest that glucose breakdown in the tadpole does not go through the phosphorylation cycle.
6. After addition of hexosediphosphate no triosephosphate accumulation in the tadpole was observed (in contrast to the chick embryo).

I wish to express my sincerest thanks to Prof. Sir F. G. Hopkins for the hospitality of his laboratory and to Dr J. Needham for his help and advice in carrying out these experiments and for suggesting this problem. I am also much indebted to Dr B. E. Holmes for lending me Warburg manometers with small cups and to Dr D. M. Needham and Dr D. E. Green for hexosediphosphate and *dl*-glyceraldehyde respectively. I am very grateful to Dr H. Lehmann for help in the method of triosephosphate estimation and for some suggestions concerning the glyceraldehyde inhibition.

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# CXX. MICRODETERMINATION OF HYPOXANTHINE AND XANTHINE

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IN the course of experiments on the formation of hypoxanthine<sup>1</sup> in pigeon liver [see Edson *et al.* 1936] it was found desirable to have a micromethod for the determination of hypoxanthine. In this paper we describe a method based on the use of xanthine oxidase which has proved satisfactory in work on surviving tissues and on enzymes. Hypoxanthine and xanthine are quantitatively oxidized to uric acid, the former requiring 2 atoms of O, the latter 1 atom. If one purine base only is present and if the nature of this is known, its concentration can be calculated from the determination of the O<sub>2</sub> uptake. If both xanthine and hypoxanthine are present their concentrations can be calculated if the uric acid formation is measured in addition to the O<sub>2</sub> uptake.

## *Preparation of xanthine oxidase*

The method requires a highly active preparation of xanthine oxidase. This is prepared in the following way [Dixon & Kodama, 1926]: fresh milk (0.5–1 l.) is clotted with a rennin preparation (Crosse and Blackwell's rennet tablets) and the clot is broken up with a glass rod in order to facilitate the separation of the whey from the curd; the whey is filtered off through muslin, one volume of milk yielding about 0.8 vol. of whey. In each 880 ml. of whey are dissolved 215 g. of solid ammonium sulphate. The solution is allowed to stand for about 30–60 min. until the globulins containing the enzyme have risen to the surface. The underlying almost clear solution is removed by suction and the surface layer amounting to less than one-fifth of the total volume is centrifuged for 10–15 min. The globulins now form a solid floating cake which is transferred to a filter paper and dried *in vacuo*. After extraction with ether to remove the fat, the ether-insoluble residue is dried again. The yield is 3–5 g. per l. of milk. The enzyme keeps *in vacuo* for several weeks. It is used in the form of a 10% solution in 0.1 M phosphate buffer of pH 7.4.

15 mg. of the enzyme (0.15 ml. 10% solution) should oxidize 0.5 mg. hypoxanthine in less than 1 hr. (40°, pH 7.4). We discarded less active preparations as the O<sub>2</sub> uptake in the presence of such preparations tended to be (up to 15%) in excess of the theoretical value. It appears that enzyme preparations of low activity are obtained from milk contaminated with inhibitors [see Philpot, 1938].

The activity of the enzyme is tested manometrically. The following solutions are required:

(1) Hypoxanthine stock solution, 12.5 mg. in 50 ml. 0.025 M phosphate buffer of pH 7.4.

(2) Aqueous pigeon liver extract: 1 g. pigeon liver is thoroughly ground with sand and 10 parts of water and centrifuged. The supernatant liquid is used. It keeps for several weeks if stored in the refrigerator and preserved with octyl alcohol.

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(3) 10 % xanthine oxidase in 0.1 *M* phosphate buffer, *pH* 7.4.

(4) Phosphate buffer, 0.1 *M*, *pH* 7.4.

In addition to the thermobarometer two conical manometric flasks with a centre chamber and a side arm are required. The first flask, serving as "blank" contains 0.5 ml. phosphate buffer, 3.5 ml. water and 0.5 ml. liver extract in the main compartment, 0.2 ml. 2*N* sodium hydroxide in the centre chamber and 0.15 ml. xanthine oxidase solution in the side arm. The second flask contains 2 ml. hypoxanthine solution, 1.5 ml. water, 0.5 ml. phosphate buffer and 0.5 ml. liver extract in the main compartment. The centre chamber and side arm are filled as in the control. The bath temperature is 40°. The side arms are mixed when thermal equilibrium is reached and readings are taken at 10 min. intervals. Suitable enzyme preparations yield figures of the following order:

O <sub>2</sub> uptake after 10 min.	20 $\mu$ l.
" 20 min.	48 $\mu$ l.
" 30 min.	81 $\mu$ l.
" 40 min.	82.5 $\mu$ l.
" 60 min.	82.5 $\mu$ l.

(Calculated: 82.4  $\mu$ l.)

The O<sub>2</sub> uptake should be complete in less than 60 min. and should agree with the theoretical value within  $\pm 2\%$ .

#### *Determination of uric acid*

We used two methods for the determination of uric acid formed in the presence of xanthine oxidase: the method of Edson & Krebs [1936] based on the conversion of uric acid into urea, and Kern & Stransky's [1937] modification of the colorimetric method of Folin. The latter method, though less specific, is more convenient and is therefore preferable when substances interfering with the colour formation are absent. This was the case in our experiments and we have therefore used the colorimetric method in most determinations.

The method of Edson & Krebs can be employed without modification. If the colorimetric method is used the concentration of uric acid in the sample must be of the same order as in blood filtrates for which the method was originally designed. It is therefore essential to dilute the solutions if the concentration of uric acid is relatively high. The determination of the O<sub>2</sub> uptake in the presence of xanthine oxidase indicates the order of magnitude of the uric acid present and we have used this guide in the following way.

If the O<sub>2</sub> uptake is below 25  $\mu$ l., the solution is rinsed with water into a 50 ml. measuring flask and water is added to fill about three-quarters of the flask. Then are added 1/100th volume of acetone (0.5 ml.), 1/25th vol. of 10 % Na<sub>2</sub>WO<sub>4</sub> (2 ml.) and 1/25th vol. of 0.66 *N* H<sub>2</sub>SO<sub>4</sub>. The flask is filled up to the mark and the contents filtered after 20 min. or more.

If the O<sub>2</sub> uptake is above 25  $\mu$ l. the dilution is increased according to the following scale:

If the O <sub>2</sub> uptake is 25–50 $\mu$ l.	dilute to 100 ml.
" 50–100 $\mu$ l.	dilute to 200 ml.
" 100–200 $\mu$ l.	dilute to 400 ml., etc.

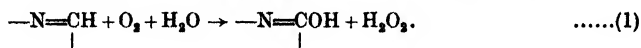
The relative proportions of acetone and deproteinizing reagents remain the same.

20 ml. filtrate are mixed with 5 ml. Na silicate solution (22 g. Na silicate cryst. Schering-Kahlbaum and 15 g. glycerol in 100 ml.) and 1 ml. Folin's phosphotungstic acid [see Kern & Stransky, 1937]. The colorimetric reading is taken after 5 min.

The standard uric acid solution is treated at the same time and in the same way as the unknown solutions: a known quantity of uric acid (about 0.03 mg.) is transferred into a 100 ml. flask and mixed with water and the reagents used in the xanthine oxidase experiments (xanthine oxidase, liver extract, buffer, acetone, tungstate,  $\text{H}_2\text{SO}_4$ ) and filtered. 20 ml. of the filtrate are used.

#### *Quantitative oxidation of purine bodies*

*Effect of liver extract.* Hypoxanthine, xanthine and purine (see later) react with  $\text{O}_2$  in the presence of xanthine oxidase as follows [Thurlow, 1925]:



The  $\text{H}_2\text{O}_2$  formed inactivates and destroys the enzyme and unless it is removed reaction (1) does not go to completion. We found in accordance with Dixon [1925] and Dixon & Keilin [1936] that quantitative yields in respect to  $\text{O}_2$  uptake and also to uric acid formation are obtained if pigeon liver extract, containing catalase, is added to xanthine oxidase. Mammalian liver is not suitable because it may contain uricase. Of the pigeon liver extract 0.5 ml. is added to 3–5 ml. solution; with smaller quantities we sometimes found incomplete uric acid yields though the  $\text{O}_2$  uptake was correct.

Data on the  $\text{O}_2$  uptake and uric acid formation in the presence of liver extract are given in Table I. The manometric arrangement was as described for the determination of xanthine oxidase activity.

Table I. *Oxidation of purine bodies in the presence of xanthine oxidase and liver extracts*

O <sub>2</sub> consumption and uric acid formation					
Substances added	Quantity		O <sub>2</sub> uptake (μl.)	Uric acid formation (μl.)	Method used for uric acid determination
	mg.	In equivalents of μl. O <sub>2</sub> *			
Hypoxanthine	0.918	151.2	150	150.5	Edson & Krebs [1936]
"	0.612	100.8	101	99.5	"
"	0.306	50.4	49.3	52	"
"	0.153	25.2	25.7	25.8	"
"	1.028	169.0	174.5	172.5	Colorimetric
"	0.514	84.5	87.0	83.5	"
"	0.257	42.3	42.5	43.0	"
"	0.129	21.1	18.0	21.7	"
Xanthine	0.206	15.2	17.4	28.9	"
"	0.412	30.4	29.7	59.7	"
"	0.440	32.4	31.6	64.4	"
Purine	0.476	132.5	133.5	86.2	"

\* Calculated on the assumption that hypoxanthine requires  $10\text{O}_2$ , xanthine  $\frac{1}{2}\text{O}_2$  per mol.

#### *Preparation of the solution for the determination of hypoxanthine and xanthine*

The sample of purine base to be determined should be contained in a small volume of liquid (5–10 ml.) suitable for the manometric determination of the  $\text{O}_2$  uptake. The reaction of the solution has to be adjusted with phosphate to

c. pH 7.4, but since the activity of xanthine oxidase does not vary much between pH 6.8 and 7.8, the adjustment need not be very accurate. The balanced salt solution of Krebs & Henseleit [1932] containing 0.025 *M* NaHCO<sub>3</sub> is adjusted to the correct pH by addition of 0.2 ml. 0.5 *M* KH<sub>2</sub>PO<sub>4</sub> to 3 ml. saline.

If the purine bases are present in a volume too large for the manometric experiments, or if interfering substances (enzyme inhibitors) are present, it is advisable to precipitate the purines by means of the copper-bisulphite method and to redissolve them in a smaller volume. [See Krüger & Schmid, 1905; Thannhauser & Czoniczer, 1920; Kerr & Blish, 1932.]

### Calculations

Let  $x_{O_2}$  = mol. O<sub>2</sub> absorbed in the presence of xanthine oxidase.  
 $x_{\text{uric acid}}$  = mol. uric acid formed in the presence of xanthine oxidase.  
 $x_{\text{Hypoxanthine and xanthine}}$  = mol. hypoxanthine and xanthine present.

If hypoxanthine requires 1 mol. and xanthine 0.5 mol. O<sub>2</sub> for the conversion into uric acid (as is the case under suitable conditions):

$$x_{\text{Hypoxanthine}} = 2x_{O_2} - x_{\text{uric acid}},$$

$$x_{\text{xanthine}} = 2(x_{\text{uric acid}} - x_{O_2}).$$

If there are other substances in addition to xanthine and hypoxanthine which absorb O<sub>2</sub> or produce uric acid in the presence of xanthine oxidase the formulae are not valid and the method is not applicable to the individual determination of hypoxanthine and xanthine. In this case, however, the method allows the calculation of the total purine bodies:

$$x_{\text{Total purine bodies}} = x_{\text{uric acid}}.$$

### Specificity of the method

*Substances absorbing O<sub>2</sub> in the presence of xanthine oxidase.* The specificity of xanthine oxidase, on which the method depends has recently been reviewed and examined by Booth [1938]. The enzyme absorbs O<sub>2</sub> in the presence of aldehydes and of a number of purine bodies. Among the purines Coombs [1927] found hypoxanthine, xanthine, 6:8-dihydroxypurine, 2-thioxanthine and adenine to react with the enzyme. Booth [1938] added 8-hydroxypurine and 2:8-dihydroxypurine to the list. Through the courtesy of Dr H. O. L. Fischer, Toronto, we were able to test another substance, purine, prepared by E. Fischer [1898]. We found that it reacts about as rapidly as hypoxanthine, yielding the theoretical amount of uric acid (Table II).

Table II. *Oxidation of purine in the presence of xanthine oxidase*

(Experimental conditions as described for testing the activity of xanthine oxidase; figures corrected for blanks.)

Substrate	0.476 mg. purine	0.514 mg. hypoxanthine	0.440 mg. xanthine
μl. O <sub>2</sub> absorbed after 20 min.	29.2	31.2	18
„ 40 min.	60.6	66.4	30
„ 60 min.	89.2	86.5	31.6
„ 120 min.	132.5	86.5	31.6
μl. O <sub>2</sub> calculated for oxidation to uric acid	133.5	85	32.4
mg. uric acid formed	0.646	0.615	0.483
mg. uric acid calc.	0.666	0.636	0.486
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It follows that an  $O_2$  uptake observed in the presence of xanthine oxidase does not finally prove the presence of xanthine or hypoxanthine. But of all the substances which may absorb  $O_2$  in the presence of xanthine oxidase, only hypoxanthine, xanthine, adenine and a few aldehydes are known to occur in biological material. Uric acid determinations show whether aldehydes or purines are present: the presence of aldehydes is indicated if an absorption of  $O_2$  but no formation of uric acid occurs. We have met with no such case in experiments on tissue extracts and body liquids, and we think it unlikely that aldehydes are found in animal tissues in quantities sufficient to interfere with the determination of purine bodies.

Adenine, if present, will be determined as hypoxanthine; its presence is suggested if the rate of oxidation is comparatively slow. Hypoxanthine reacts about 20 times faster than adenine [Booth, 1938].

*Substances liberating hypoxanthine or xanthine.* Nucleotides, nucleosides or guanine gradually liberate hypoxanthine or xanthine under the conditions of the determination. Among these potential precursors of purine bases muscle adenylic acid, free or as dinucleotide, is quantitatively the most important one in animal tissues. The method as described in this paper, does not distinguish between free and "bound" purine bodies (Table III). To differentiate between these, xanthine oxidase and catalase preparations free from nucleotidase, nucleosidase and guanase must be used. In the material, however, for which the method was designed, viz. pigeon liver, the concentration of bound purine is negligible as compared with the amount of free hypoxanthine produced and we have therefore made no attempts to prepare pure xanthine oxidase and catalase.

Table III.  $O_2$  uptake in the presence of adenylic acid and of guanine

40 mg. "xanthine oxidase"; 0.5 ml. liver extract 1 : 10; 4 ml. *M*/40 phosphate buffer.

Substrate added:	4.25 mg. muscle adenylic acid	4.85 mg. yeast adenylic acid	2.1 mg. guanine	0.464 mg. hypoxanthine
$\mu$ l. $O_2$ absorbed after 20 min.	6	2	6	20
" 40 min.	16	5	11	72
" 140 min.	63	27	26	77
" 250 min.	91	42	38	77

#### *Interfering substances*

A number of substances interfere with the quantitative determination of the  $O_2$  uptake because they undergo a "coupled oxidation" under the conditions of the determination. Such substances are nitrites [ThurLOW, 1925] *p*-phenylenediamine [Harrison & ThurLOW, 1926], methaemoglobin [Harrison & ThurLOW, 1926; Dixon & Keilin, 1936], methyl, ethyl and propyl alcohols [Keilin & Hartree, 1936] and haemoglobin [Krebs, 1936; Bernheim *et al.* 1936]. Among these, haemoglobin and its derivatives are the only substances likely to be present in animal tissues. We find, however, that the traces of haemoglobin present in tissue extracts or in solutions in which slices have been kept do not affect the  $O_2$  consumption if liver extract is present. Liver extract inhibits the coupled oxidation of haemoglobin and methaemoglobin (Table IV) although it promotes the coupled oxidation of alcohol [Keilin & Hartree, 1936]. If the concentration of haemoglobin or methaemoglobin is very high the inhibition of the coupled oxidation is not always complete. In this case, or in the presence

of alcohol, it is not possible to calculate the concentrations of hypoxanthine and xanthine from the  $O_2$  uptake and only the concentration of the total purine bodies can be measured under these conditions.

Table IV. *Effect of liver extract on the "coupled oxidation" of haemoglobin*

Each flask contained 0.44 mg. hypoxanthine in 3 ml. 0.05 *M* phosphate buffer, pH 7.4; 15 mg. xanthine oxidase in 0.15 ml. The figures have been corrected for blanks. The haemoglobin solution was prepared by cytolysing washed pigeon red cells with 10 vol. of water. The solution was cleared by centrifuging. 1 ml. was equivalent to 108  $\mu$ l.  $O_2$ .

Added to solution:		0.1 ml. haemo- globin solution	0.1 ml. haemo- globin solution; 0.5 ml. pigeon liver extract	0.5 ml. pigeon liver extract
$\mu$ l. $O_2$ absorbed after	10 min.	28	48	22
"	20 min.	46	98.5	66
"	30 min.	56	128	70
"	40 min.	64	130.5	71
"	50 min.	66	132	72
Calc. for 1 mol. $O_2$		72.6	72.6	72.6

#### SUMMARY

1. Conditions have been ascertained under which xanthine oxidase brings about the quantitative conversion of hypoxanthine and xanthine into uric acid.

2. A method based on the use of xanthine oxidase is described which permits the determination of hypoxanthine and xanthine in quantities from 0.1 mg. upwards. If both  $O_2$  uptake and uric acid formation are measured it is possible to distinguish between hypoxanthine and xanthine, since the former requires 2 atoms, the latter 1 atom of oxygen.

3. In testing the specificity of the method it was found that purine reacts with xanthine oxidase as rapidly as hypoxanthine, but since purine has never been found in biological material it is improbable that it will interfere.

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# CXXI. THE FORMATION OF HYPOXANTHINE IN PIGEON LIVER

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EDSON *et al.* [1936] isolated hypoxanthine from solutions in which pigeon liver had been allowed to metabolize ammonium salts. Reindel & Schuler [1937] on the other hand, using alanine as substrate, found only traces of hypoxanthine and claimed that xanthine is the chief purine base synthesized in pigeon liver. In order to elucidate this discrepancy we re-examined the nature of the purine base formed using the method described in the preceding paper. In our former experiments hypoxanthine was isolated as hypoxanthine-AgNO<sub>3</sub> but the yields were not quantitative owing to unavoidable losses during the isolation and purification. The isolation experiments therefore left it open whether some xanthine is formed in addition to hypoxanthine. The new method enabled us to determine both purine bases quantitatively. The experiments confirm our previous results: hypoxanthine is virtually the only purine base synthesized in pigeon liver. Although the experimental conditions were widely varied we were unable to find significant quantities of xanthine.

The second part of the paper deals with the rate of hypoxanthine synthesis under varying conditions. Oxaloacetate and glutamine were found to increase specifically the rate of hypoxanthine synthesis. These substances may therefore be assumed to play a special part in the hypoxanthine synthesis, the nature of which, however, remains to be investigated.

## *Methods*

The experiments were carried out in conical manometric flasks. Slices of pigeon liver were shaken at 40° in a saline medium containing 0.025 *M* NaHCO<sub>3</sub>-CO<sub>2</sub> or phosphate buffer. Various substrates were added in the form of 0.2 *M* neutral solutions. After 1 or 2 hr. the slices were removed from the solution. If NaHCO<sub>3</sub>-saline was used, 0.2 ml. 0.5 *M* KH<sub>2</sub>PO<sub>4</sub> was added to the solution and 0.2 ml. 2*N* NaOH was placed in the centre cup of the manometric flask. The flask was then shaken for 20 min. in the water bath in order to remove the CO<sub>2</sub> from the solution. The flask was again disconnected; 1.0 ml. 0.1 *M* phosphate buffer of pH 7.4 and 0.5 ml. liver extract were placed in the main compartment and 0.2 ml. 10% xanthine oxidase in the side-arm. If the medium was phosphate saline the addition of KH<sub>2</sub>PO<sub>4</sub> and the subsequent shaking for 20 min. were omitted. The O<sub>2</sub> uptake and uric acid formation were determined as described in the preceding paper. A blank determination on the reagents was carried out in all experiments.

Added hypoxanthine and xanthine were quantitatively recovered under these conditions.

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*Nature of the purine base synthesized in pigeon liver*

Some experiments in which the substrates and media were varied are recorded in Table I. The ratio  $\frac{\text{uric acid}}{\text{O}_2}$  (last column) is, within the limits of error, 1.0, as is expected for hypoxanthine. The deviations are insignificant and in both directions. This leaves no doubt that the bulk of the purine base formed in pigeon liver is hypoxanthine.

Table I. *Determination of the purine base formed in pigeon liver*

40°. Period of incubation 60 min.

Substrate (final conc.)	Gas	Tissue mg.	In presence of xanthine oxidase		Ratio $\frac{\text{uric acid}}{\text{O}_2}$
			$\mu\text{l. O}_2$ absorbed	$\mu\text{l. uric}$ acid formed	
NaHCO <sub>3</sub> saline:					
—	5% CO <sub>2</sub> in O <sub>2</sub>	27.7	24.9	24.0	0.96
l(+)Alanine (0.02 M)	..	24.8	31.3	28.7	0.95
l(+)Glutamic acid (0.02 M)	..	25.6	25.2	24.2	0.96
l(+)Glutamine (0.02 M)	..	26.0	50.8	52.6	1.04
l(−)Aspartic acid (0.02 M)	..	29.0	31.2	28.2	0.91
l(−)Asparagine (0.02 M)	..	28.9	39.2	42.8	1.09
0.003 M NH <sub>4</sub> Cl; 0.02 M pyruvate	..	22.6	39.0	36.4	0.93
0.003 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate	..	27.1	53.1	55.9	1.05
0.02 M Oxaloacetate; 0.02 M l(+) glutamine	..	21.6	55.0	56.4	1.02
—	..	14.06	15.0	13.3	0.89
—	5% CO <sub>2</sub> in N <sub>2</sub>	44.04	12.5	11.2	0.90
0.04 M Pyruvate; 0.02 M NH <sub>4</sub> Cl	5% CO <sub>2</sub> in O <sub>2</sub>	17.04	36.1	33.2	0.91
0.04 M Oxaloacetate; 0.02 M NH <sub>4</sub> Cl	..	16.12	38.5	34.7	0.90
0.02 M Oxaloacetate; 0.01 M NH <sub>4</sub> Cl	5% CO <sub>2</sub> in N <sub>2</sub>	44.77	14.7	12.2	0.84
Phosphate saline:					
0.02 M Pyruvate; 0.01 M NH <sub>4</sub> Cl	O <sub>2</sub>	14.90	13.7	13.0	0.95
0.02 M Oxaloacetate; 0.01 M NH <sub>4</sub> Cl	..	9.57	23.5	22.8	0.97

*Rate of hypoxanthine synthesis*

Since hypoxanthine is the only purine base formed in pigeon liver, its concentration in the medium may be calculated directly from the amount of O<sub>2</sub> absorbed in the presence of xanthine oxidase. We have therefore dispensed with the determination of the uric acid formation in the further experiments which were carried out to measure the rate of hypoxanthine formation under varying conditions. Eleven sets of typical experiments are recorded in Table II. The rates are expressed by the quotient:

$$Q_{\text{hypoxanthine}} = \frac{\mu\text{l. O}_2 \text{ absorbed in the presence of xanthine oxidase}}{\text{mg. (dry) liver used} \times \text{hr. of incubation}}$$

These experiments show the following:

(1) There is a small formation of hypoxanthine when no substrate is added (last column). This "blank" is still much smaller under anaerobic conditions (Exp. 4). It is probable that the hypoxanthine formed anaerobically does not arise from synthesis but from the breakdown of adenine nucleotides.

(2) Addition of amino-acid causes an increase of the rate of hypoxanthine synthesis (Exps. 1, 9). Among the substances tested glutamine has the largest effect (Exps. 1, 2, 3, 4, 5, 11). The increase does not occur under anaerobic conditions.

(3) NH<sub>4</sub>Cl causes an increase of the rate of synthesis if added in low concentrations (0.005 M). In higher concentrations it tends to inhibit (Exp. 5).



Table II. *Rate of hypoxanthine synthesis in pigeon liver at 40°*

Table 11. Rate of hypoxanthine synthesis in pigeon liver at 35°C			Q hypoxanthine	
No.	Gas	Substrates (final conc.)	With	No
			substrate	substrate
NaHCO <sub>3</sub> saline				
1	5% CO <sub>2</sub> in O <sub>2</sub>	0.02 M l(+)Alanine	1.26	0.90
	"	0.02 M l(+)Glutamine	1.95	0.90
	"	0.02 M l(+)Glutamate	0.99	0.90
	"	0.02 M l(-)Aspartate	1.07	0.90
	"	0.02 M l(-)Asparagine	1.35	0.90
	"	0.02 M l(-)Arginine	0.81	0.90
	"	0.0033 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate	1.97	0.90
	"	0.02 M l(+)Glutamine; 0.02 M oxaloacetate	2.54	0.90
2	"	0.0033 M NH <sub>4</sub> Cl; 0.02 M pyruvate	1.72	0.90
	"	0.01 M l(+)Glutamine	1.79	1.03
3	"	0.01 M l(+)Glutamine	1.31	0.71
	"	0.0066 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate	2.60	0.71
	"	" 0.02 M succinate	1.06	0.71
	"	" 0.02 M l(-)malate	0.82	0.71
4	5% CO <sub>2</sub> in N <sub>2</sub>	" 0.02 M acetoacetate	0.92	0.71
	5% CO <sub>2</sub> in O <sub>2</sub>	0.01 M l(+)Glutamine	0.28	0.30
		0.0033 M NH <sub>4</sub> Cl	0.26	0.30
		0.0033 M NH <sub>4</sub> Cl; 0.02 M pyruvate	0.31	0.30
		0.01 M Glutamine	1.08	0.89
		0.0033 M NH <sub>4</sub> Cl	0.64	0.89
5	"	0.0033 M NH <sub>4</sub> Cl; 0.02 M pyruvate	1.20	0.89
	"	0.01 M l(+)Glutamine	1.01	0.36
	"	0.02 M l(+)Glutamine	1.12	0.36
	"	0.04 M l(+)Glutamine	1.21	0.36
	"	0.005 M NH <sub>4</sub> Cl	0.70	0.36
	"	0.01 M NH <sub>4</sub> Cl	0.53	0.36
	"	0.02 M NH <sub>4</sub> Cl	0.44	0.36
6	"	0.01 M NH <sub>4</sub> Cl	0.79	1.07
	"	0.01 M NH <sub>4</sub> Cl; 0.02 M pyruvate	2.12	1.07
	"	0.01 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate	2.34	1.07
7	"	0.01 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate	1.83	0.72
	"	0.01 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate; 0.01 M l(+)alanine	1.72	0.72
	"	0.01 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate; 0.01 M l(+)ornithine	2.11	0.72
	"	" 0.01 M l(+)arginine	1.94	0.72
	"	" 0.01 M dl-serine	1.49	0.72
	"	" 0.01 M l(-)asparagine	1.85	0.72
	"	" 0.01 M l(-)aspartate	1.61	0.72
Phosphate saline				
8	O <sub>2</sub>	0.01 M NH <sub>4</sub> Cl	0.67	0.63
	"	0.01 M NH <sub>4</sub> Cl; 0.02 M pyruvate	0.92	0.63
	"	0.01 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate	2.45	0.63
NaHCO <sub>3</sub> saline				
9	5% CO <sub>2</sub> in O <sub>2</sub>	0.01 M l(+)Ornithine	1.36	0.45
	"	0.01 M l(-)Leucine	1.20	0.45
	"	0.01 M l(+)Valine	0.99	0.45
	"	0.02 M dl-Lysine	0.80	0.45
	"	0.01 M l(-)Methionine	0.94	0.45
	"	0.01 M l(-)Histidine	1.01	0.45
	"	0.01 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate	1.41	0.52
10	"	0.02 M glucose	0.78	0.52
	"	0.5% glycogen	1.05	0.52
	"	0.02 M citrate	1.26	0.52
	"	0.02 M α-glycerophosphate	1.26	0.52
	"	0.02 M l(+)lactate	0.56	0.52
	"	0.02 M pyruvate	0.82	0.52
	"	"	1.55	0.81
11	"	0.02 M citrate	1.44	0.81
	"	0.02 M oxaloacetate	2.62	0.81
	"	0.02 M pyruvate	1.92	0.81
	"	0.02 M l(+)Glutamine	1.67	0.81
	"	0.02 M l(+)Glutamine; 0.01 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate	3.40	0.81
	"	0.02 M l(+)Citrulline	0.92	0.81
	"	0.02 M l(+)Citrulline; 0.01 M NH <sub>4</sub> Cl; 0.02 M oxaloacetate	2.38	0.81

(4) Of the non-nitrogenous substances (when added together with  $\text{NH}_4\text{Cl}$ ) oxaloacetate effects the greatest increase of the rate of hypoxanthine synthesis (Exps. 1, 3, 6, 8, 10).

(5) When glutamine,  $\text{NH}_4\text{Cl}$  and oxaloacetate are added together a summation of the effects sometimes occurs (Exp. 11).

(6) Amino-acids, when added together with  $\text{NH}_4\text{Cl}$  and oxaloacetate, have no significant effect on the hypoxanthine synthesis (Exps. 7, 11). There is thus no effect comparable with the action of ornithine on the urea synthesis in rat liver. This negative result does exclude the possibility that the substances tested play a part in the hypoxanthine synthesis, as a positive effect can only be expected if the concentration of the substance tested is a "limiting factor".

#### *Hypoxanthine formation in other tissues*

The livers of the fowl, duck, rat and guinea-pig were treated in the same way as pigeon liver, but no appreciable quantities of hypoxanthine were found when the conditions were aerobic. This may be expected since added hypoxanthine disappears in these tissues; they all contain xanthine oxidase. Anaerobically small quantities of hypoxanthine were liberated in these tissues,  $Q_{\text{hypoxanthine}}$  being of the order 0.2.

#### *Effects of glutamine and oxaloacetate on the uric acid synthesis in fowl liver*

In view of the effects of oxaloacetate and glutamine on the hypoxanthine synthesis in pigeon liver we thought it of interest to investigate the effects of these substances on the corresponding reaction in fowl liver, the uric acid synthesis. Uric acid was determined colorimetrically, as described in the preceding paper.  $Q_{\text{uric acid}}$ , the rate of uric acid synthesis, is expressed in terms equivalent to  $Q_{\text{hypoxanthine}}$ . The results, recorded in Table III show that the corresponding effects do in fact occur.

Table III. *Effect of glutamine and oxaloacetate on the uric acid formation in fowl liver*

Substrates added (final concentration)	$Q_{\text{uric acid}}$
—	0.41
0.02 <i>M</i> Oxaloacetate; 0.01 <i>M</i> $\text{NH}_4\text{Cl}$	1.32
0.02 <i>M</i> l(+) Glutamine	1.33
0.02 <i>M</i> Pyruvate; 0.01 <i>M</i> $\text{NH}_4\text{Cl}$	1.04

#### DISCUSSION

*Reindel & Schuler's results.* Although no explanation can be offered for the findings of Reindel & Schuler [1937] mentioned in the introduction, it appears certain from our experiments that xanthine is not formed *synthetically* in pigeon liver. It may be possible that traces of xanthine arise *hydrolytically* from guanine, or guanine nucleosides or nucleotides, since pigeon liver contains a guanase; but the quantities of guanine compounds present in the liver are almost negligible compared with the amounts of hypoxanthine synthesized in the presence of an excess of  $\text{NH}_4$  salts.

*The effect of oxaloacetate.* The stimulating effect of oxaloacetate suggests the idea that oxaloacetate serves as the source of the carbon skeleton in the synthesis of the purine ring. Oxaloacetate, as an intermediate product in the oxidative

breakdown of carbohydrate, lactate and pyruvate belongs to the relatively small number of substances available in sufficient quantities.

*The effect of glutamine.* The facts at our disposal are not sufficient to explain the action of glutamine. It is improbable that the carbon chain of the glutamine molecule, or part of it, enters directly into the purine ring, but more likely that glutamine acts as an "ammonia carrier". It is of great interest in this connexion that Leuthardt [1938] found a somewhat analogous effect of glutamine on the urea synthesis in the liver of starved guinea-pigs.

These effects of glutamine, however, are not necessarily to be explained by a direct participation in the synthesis, since synthetic reactions are always complex processes connected with the production and transmission of energy. Several observations, such as the occurrence of a glutamine synthesis in brain cortex and in retina [Krebs, 1935] and the necessity of glutamine for the growth of *Streptococcus haemolyticus* [McIlwain *et al.* 1939], suggest that glutamine plays a more general, though still entirely undefined, role in cell metabolism.

#### SUMMARY

1. The purine base synthesized by pigeon liver requires 1 mol. O<sub>2</sub> for its quantitative conversion into uric acid. The base is therefore hypoxanthine. Contrary results of Reindel & Schuler [1937] who claim to have found a formation of xanthine remain unexplained.

2. The effects of added substrates on the rate of hypoxanthine synthesis were studied. Glutamine and oxaloacetate were found to increase the rate of the synthesis. The cause of these effects is discussed.

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## CXXII. THE SYNTHESIS OF GLUTAMINE IN PIGEON LIVER

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It has previously been shown [Edson *et al.* 1936] that pigeon liver utilizes added  $\text{NH}_3$  more rapidly than can be accounted for by the rate of hypoxanthine synthesis. Under some conditions, for instance in the presence of 0.02 to 0.04 *M* ammonium pyruvate, the amount of  $\text{NH}_3$  converted into hypoxanthine is less than 20 % of total  $\text{NH}_3$  metabolized; the bulk of the  $\text{NH}_3$  is converted into substances which remained unidentified. We have now identified glutamine in this fraction. Its quantity accounts for about 40 to 50 % of the total  $\text{NH}_3$  metabolized.

### EXPERIMENTAL

As in the previous experiments slices of pigeon liver suspended in the saline medium of Krebs & Henseleit [1932] were used. Small scale experiments were carried out in manometric flasks, large scale experiments in "metabolism flasks", as described by Krebs [1933]. These flasks have a diameter of about 11 cm. and they contained not more than 100 ml. medium in order to secure sufficient oxygenation. The temperature was 40°. Ammonia was determined according to Parnas & Heller [1924], amino-N according to Van Slyke [1929] after the removal of  $\text{NH}_3$  from the solution.

#### *Formation of "amino-nitrogen" in pigeon liver*

Data concerning the rate of  $\text{NH}_3$  uptake have already been given [Edson *et al.* 1936] and it has also been mentioned that part of the  $\text{NH}_3$  is converted into amino compounds. In a series of further experiments we compared the rates of  $\text{NH}_3$  uptake and of hypoxanthine and "amino-N" formation. The highest rates of  $\text{NH}_3$  uptake and amino-N formation were observed in the presence of pyruvate. An example is given in Table I. In this experiment the amino-N formed accounts for most of the metabolized  $\text{NH}_3$ . "Amino-N", as determined by Van Slyke's method, it should be remembered, does not necessarily imply  $=\text{CHNH}_2$  groups but would include among other groupings the  $-\text{CONH}_2$  group of glutamine.

Table I. *Utilization of added  $\text{NH}_4\text{Cl}$  by pigeon liver*

The medium contained 0.04 *M* pyruvate and 0.01 *M*  $\text{NH}_4\text{Cl}$ ; 44 mg. liver; 2 hr.; 40°.

mg. $\text{NH}_3$ -N added	0.840
mg. $\text{NH}_3$ -N used	0.795
mg. hypoxanthine-N formed	0.110
mg. "amino-N"	0.658

Data for control to which no  $\text{NH}_4\text{Cl}$  was added calculated for same quantity of tissue.

mg. $\text{NH}_3$ -N formed	0.021
mg. hypoxanthine-N formed	0.081
mg. amino-N formed	0.163

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*Formation of amide-nitrogen*

If the solution from which  $\text{NH}_3$  has been removed by pigeon liver is heated for 10 min. to  $100^\circ$  in the presence of 5%  $\text{H}_2\text{SO}_4$ , 15 to 25% of the  $\text{NH}_3$  metabolized returns to the solution. Examples are given in Table II. Heating for

Table II.  $\text{NH}_3$ -consumption and amide formation in pigeon liver

No.	Substrate added (total volume of medium 3-4 ml.)	mg. tissue	Time min.	$\mu\text{l. NH}_3$ used	$\mu\text{l. amide-N}$ formed	$Q_{\text{NH}_3}$	$Q_{\text{amide-N}}$
1	—	29.9	90	+ 26	39.6	+ 0.58	+ 0.88
	0.01 $M$ $\text{NH}_4\text{Cl}$ ; 0.04 $M$ pyruvate	29.6	90	— 792	131	— 17.9	+ 2.95
	0.01 $M$ $\text{NH}_4\text{Cl}$ ; 0.02 $M$ $l(+)$ glutamate	40.2	90	— 412	115	— 6.8	+ 1.91
	0.01 $M$ $\text{NH}_4\text{Cl}$ ; 0.01 $M$ $\alpha$ -ketoglutarate	36.0	90	— 447	113	— 8.3	+ 2.00
2	0.0066 $M$ $\text{NH}_4\text{Cl}$	16.1	60	— 118	30	— 7.35	+ 1.87
	0.0066 $M$ $\text{NH}_4\text{Cl}$ ; 0.02 $M$ pyruvate	13.0	60	— 267	42.5	— 20.5	+ 3.28
	0.0066 $M$ $\text{NH}_4\text{Cl}$ ; 0.02 $M$ oxaloacetate	10.9	60	— 186	31.7	— 17.1	+ 2.92
3	0.01 $M$ $\text{NH}_4\text{Cl}$	32.5	90	— 304	— 76.5	— 6.25	+ 1.57
	0.01 $M$ $\text{NH}_4\text{Cl}$ ; 0.02 $M$ $\alpha$ -ketoglutarate	30.7	90	— 327	100	— 7.1	+ 2.17
	0.01 $M$ $\text{NH}_4\text{Cl}$ ; 0.02 $M$ $l(+)$ glutamate	27.8	90	— 266	85.5	— 6.4	+ 2.05
	0.01 $M$ $\text{NH}_4\text{Cl}$ ; 0.02 $M$ pyruvate	28.7	90	— 618	145	— 14.4	+ 3.37
4	0.02 $M$ $\text{NH}_4\text{Cl}$	68.7	120	— 182	98	— 1.3	+ 0.71
	0.02 $M$ $\text{NH}_4\text{Cl}$ ; 0.02 $M$ pyruvate	73.2	120	— 1070	198	— 7.3	+ 1.35
	0.02 $M$ $\text{NH}_4\text{Cl}$ ; 0.02 $M$ oxaloacetate	61.0	120	— 800	157	— 6.6	+ 1.28
5	0.02 $M$ $\text{NH}_4\text{Cl}$	52.5	100	— 658	144	— 7.5	+ 1.6
	0.02 $M$ $\text{NH}_4\text{Cl}$ ; 0.02 $M$ pyruvate	38.4	100	— 1090	257	— 17.0	+ 4.0
	0.02 $M$ $\text{NH}_4\text{Cl}$ ; 0.04 $M$ pyruvate	37.3	100	— 1122	313	— 18.1	+ 5.0
	0.02 $M$ $\text{NH}_4\text{Cl}$ ; 0.04 $M$ $l(+)$ lactate	17.6	100	— 419	146	— 5.4	+ 1.84
	0.02 $M$ $\text{NH}_4\text{Cl}$ ; 0.04 $M$ $l(+)$ glutamate	42.0	100	— 589	248	— 8.4	+ 3.55
	0.02 $M$ $\text{NH}_4\text{Cl}$ ; 0.04 $M$ $\alpha$ -ketoglutarate	44.0	100	— 574	148	— 7.0	+ 2.28

more than 10 min. does not significantly increase the yield of  $\text{NH}_3$ . Alkaline hydrolysis (10 min.,  $100^\circ$ , 2N NaOH) liberates the same quantity of  $\text{NH}_3$  as acid hydrolysis. The solutions thus contain a compound which behaves like glutamine or similar acid amides [*vide* Krebs, 1935, 1].

The yield of amide-N is greatest in the presence of pyruvate. There is only a small increase over the blank when  $l(+)$ glutamate or  $\alpha$ -ketoglutarate is added. In this respect pigeon liver behaves differently from a group of tissues comprising mammalian brain and retina and the kidney of guinea-pig and rabbit in which a synthesis of glutamine was previously found to take place [Krebs, 1935]. In these tissues a rapid synthesis of glutamine occurs if ammonium glutamate is added. Ammonium pyruvate (and alanine) are also converted into glutamine but the rate of the synthesis is in this case no more than 10 or 20% of that observed in the presence of ammonium glutamate (Table III).

Table III. *Formation of glutamine in guinea-pig kidney*

$40^\circ$ ; bicarbonate saline; 0.01  $M$   $\text{NH}_4\text{Cl}$ . 5%  $\text{CO}_2$  in  $\text{O}_2$ .

Substrates (final concentration)	$Q_{\text{amide-N}}$
—	1.31
0.02 $M$ pyruvate	2.40
0.02 $M$ $l(+)$ glutamate	12.9
0.02 $M$ $l(+)$ alanine	4.7
0.03 $M$ $\alpha$ -ketoglutarate	3.6
0.02 $M$ $\alpha$ -ketoglutarate; 0.02 $M$ $l(+)$ alanine	10.3

*Isolation of the acid amide*

The determination of amide-N on samples containing 0.05–0.1 mg. amide-N proved a convenient guide in the isolation of the amide. The amide compound is not precipitated by phosphotungstic acid, Cu or Ag salts, but forms an

insoluble compound with mercuric salts in weakly acid, neutral or weakly alkaline solution. After many preliminary tests the isolation was finally carried out in the following way.

Two or three livers of well fed, freshly killed pigeons are roughly weighed and then sliced with a razor. For each g. fresh weight 7 ml. saline [Krebs & Henseleit, 1932], 0.7 ml. 0.2 *M*  $\text{NH}_4\text{Cl}$  and 1.4 ml. 0.2 *M* sodium pyruvate are measured into a metabolism flask [Krebs, 1933]. The proportion of  $\text{NH}_4\text{Cl}$  to tissue chosen allows an almost complete utilization of the added  $\text{NH}_3$  during an experimental period of 3 hr. The removal of  $\text{NH}_3$  simplifies the purification of the substance.

The flasks are filled with 5%  $\text{CO}_2$  in  $\text{O}_2$  and shaken at  $40^\circ$  for 3 hr. at the rate of 40–60 periods per min. After shaking 0.2 vol. 30% trichloroacetic acid is added and the mixture is cooled in ice. It is filtered after about 30 min. A slight turbidity of the filtrate does not interfere with the later stages of the isolation and may be neglected. The filter is washed with 3% trichloroacetic acid.

The acid amide is then precipitated with the mercuric nitrate reagent introduced by Schulze [1882]. To each 50 ml. of the combined filtrate and washings are added 5 ml. of mercuric oxide, dissolved in nitric acid (160 ml. concentrated  $\text{HNO}_3$ , 300 ml.  $\text{H}_2\text{O}$  and 220 g.  $\text{HgO}$  are boiled under reflux until the oxide is dissolved. After cooling *N*  $\text{NaOH}$  is added to produce a faint opalescence. The volume is made up to 1 l. with  $\text{H}_2\text{O}$ ).

A 30%  $\text{NaOH}$  solution is slowly added under constant shaking until the solution is distinctly alkaline (litmus). It is then placed in the refrigerator overnight. The precipitate is centrifuged and the supernatant is tested with a drop of  $\text{NaOH}$ . If a yellow precipitate is formed more  $\text{NaOH}$  is added. The precipitate which contains among other substances the mercury salts of the "amide" is washed twice on the centrifuge with distilled water. After the last washing the precipitate is suspended in water and treated with  $\text{H}_2\text{S}$ . The mercuric sulphide is filtered off and washed with water. The filtrate is concentrated *in vacuo* to about 2 or 3 ml., transferred into a centrifuge tube and dried in a vacuum desiccator.

The oily or dry residue is stirred with about 0.5 ml. water, 0.1 ml. conc.  $\text{HCl}$  and 3 ml. methyl alcohol; if an insoluble fraction remains, it is removed by centrifuging; the supernatant is mixed with sufficient ether to produce a turbidity. Crystals appear soon when the mixture is placed in the ice box. Addition of more ether increases the yield, but sometimes lowers the purity of the product. Yield: 30–50 mg. per 10 g. wet liver. The substance is recrystallized by dissolving it in a few drops of water, adding 4 vol. methyl alcohol and ether as described above.

The combined yields from several experiments were recrystallized until the melting point remained constant at  $171\text{--}173^\circ$  (uncorrected). The compound begins to darken a few degrees below this temperature and decomposes when melting. The analytical data (Dr Weiler and Dr Strauss) agree with those for glutamine hydrochloride ( $\text{C}_5\text{H}_{11}\text{N}_2\text{O}_3\text{Cl}$ ):

C found:	32.91 %;	33.05 %;	calculated for $\text{C}_5\text{H}_{11}\text{N}_2\text{O}_3\text{Cl}$ :	32.86 %
H	5.68 %;	6.13 %;		6.07 %
N	15.2 %;			15.39 %
Cl	18.8 %;			19.4 %

Treatment with chloramine T under the conditions described by Cohen [1939] for the determination of glutamic acid yielded succinic acid. From 2.87 mg. of

the substance 1.1 mg. succinic acid were obtained. The yield is not quantitative but of the same order as that obtained with pure glutamine (60–70%).

A preparation of glutamine hydrochloride obtained by dissolving glutamine from mangel-wurzels in a small volume of 10% HCl and precipitation with alcohol-ether was identical in every respect with the substance isolated from pigeon liver. The mixed melting points were unchanged.

The quantities of glutamine isolated in the pure state varied from 40 to 60% of the total "amide" present in the starting material. A considerable loss during the preparation is almost unavoidable owing to the instability of glutamine, and we therefore assume that glutamine represents most, if not all, of the amide synthesized in pigeon liver.

#### *Glutamine synthesis in other species*

Fowl and duck livers also form glutamine from added ammonium pyruvate and the rate was found to be about the same as in pigeon liver. Since the isolation of glutamine was carried out in the same way, these experiments are not described in full.

Rat liver, too, produces an acid amide, but in this case the rate is not appreciably increased over the blank when ammonium pyruvate is added. The maximum rate observed in rat liver was 1.0–1.5 ( $Q_{\text{amide-N}}$ ), against values up to 5.0 in pigeon liver. The amide formed in rat liver has not yet been isolated and identified.

#### DISCUSSION

*Synthesis of glutamine from 3-carbon compounds.* The synthesis of glutamine from ammonium pyruvate involves the formation of a 5-carbon chain. It is probable that the synthesis of  $\alpha$ -ketoglutaric acid is the primary step in the formation of glutamine. Several mechanisms have previously been discussed for the synthesis of this compound [*vide* Krebs, 1935, 2; Weil-Malherbe, 1936; Krebs & Johnson, 1937] but it is yet too early to decide between the various possibilities.

*Glutamine synthesis in guinea-pig kidney and bird's liver.* It was pointed out that guinea-pig kidney (and some other tissues) synthesize glutamine more rapidly from ammonium glutamate than from ammonium pyruvate, whilst pigeon liver yields glutamine more rapidly when ammonium pyruvate is the substrate. Such differences in rates, in our view, do not necessarily indicate entirely different mechanisms; they may be explained on the assumption that the factors limiting the rate of glutamine synthesis are different in the two groups of tissues.

*Significance of the glutamine synthesis.* The significance of the synthesis of glutamine remains obscure as long as the role of glutamine in tissue metabolism is unknown. Some observations mentioned in the discussion of the preceding paper and the facts reported in this paper leave no doubt that glutamine is a factor of general importance in cell metabolism, but the nature of its function is not yet clear.

#### SUMMARY

Slices of pigeon, fowl or duck liver form glutamine from ammonium pyruvate. The substance was isolated as glutamine hydrochloride; 1 g. of liver (dry weight) produces up to 30 mg. glutamine per hour.

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# CXXIII. A METHOD FOR THE SEPARATION OF SUGARS BY THE CHROMATOGRAPHIC ADSORPTION OF THEIR COLOURED ESTERS

## I. SEPARATION OF GLUCOSE AND FRUCTOSE

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*(Received 13 April 1939)*

IN spite of the very large amount of work which has been done on the separation of individual carbohydrates from their mixtures the methods in general use are not very satisfactory if only small quantities of the substance are available. These methods depend ultimately on differences in solubilities or boiling-points of the carbohydrates or of their derivatives. The recent development of the method of chromatographic adsorption, has shown that the degree of affinity of adsorption may vary to a much greater extent with the molecular structure, than does the boiling-point or solubility. Further, the method of chromatographic adsorption can readily be adapted for microchemical work which is frequently essential in biochemical research.

From a large number of chromatographic tests carried out during the present investigation with sugars and their derivatives the best results were obtained with two groups of arylated derivatives, namely the azobenzene-*p*-benzene-sulphonyl and azobenzene-*p*-benzoyl esters. This latter group has the advantage that the sugars are more easily separated by saponification after the chromatographic separation has been effected.

For brevity azobenzene-*p*-benzoyl chloride will be termed azoyl chloride, and the corresponding esters, azoyl esters.

The azoyl chloride reacts readily in pyridine solution with the hydroxyl groups of sugars. After reaction is complete the excess of azoyl chloride is converted into the methyl ester by the addition of methanol. The methyl ester can then be readily eliminated owing to its great solubility in most organic solvents.

The azoyl esters are coloured substances varying from orange to dark red. They are adsorbed from their solutions in organic solvents by many of the adsorbents commonly employed for chromatographic adsorption. Brockmann's  $\text{Al}_2\text{O}_3$ , treated with methanol for three days at  $37^\circ$  and dried in the air, possesses good adsorbent qualities for these esters but subsequent elution is not quantitative. Better results are obtained with silica (pure precip. B.D.H.) as the adsorbent. In this case the elution of the ester can easily be made quantitative.

The high molecular weight of the azoyl group and the fact that it is possible, in small adsorption columns, to carry out the separation of a few mg. of substances, makes the use of this method possible for microchemical work. For example, using a column 7 mm. in diameter and 150 mm. high it has been possible to carry out the separation of 10 mg. of glucopyranose ester and 10 mg. of fructopyranose ester which are equivalent to less than 2 mg. of each sugar. A preliminary account of this work has already been published [Reich, 1939].

## EXPERIMENTAL

1. *Preparation of the azobenzene-*p*-benzoic acid*

This substance was prepared by Angeli & Valori [1913] by condensation of *p*-aminobenzoic acid and nitrosobenzene in glacial acetic acid. The authors give m.p. 241°. The following method has been found to give better yields. 69 g. of dry *p*-aminobenzoic acid are dissolved in 450 ml. of hot absolute alcohol and 80 ml. of glacial acetic acid are added to the hot solution. The solution is cooled to room temperature, 60 g. of thoroughly pulverized dry nitrosobenzene are added and the mixture is shaken until the latter has dissolved. The mixture is then kept for 60 hr. at room temperature, followed by 12 hr. at 0°. Most of the azobenzene-*p*-benzoic acid formed separates from the solution in crystals which are filtered off, well washed with water, dried *in vacuo* over CaCl<sub>2</sub> and recrystallized from boiling absolute alcohol, the concentrated solution being cooled slowly to 0°. The substance crystallizes as mats of red needles with a golden glint. A further portion of the pure substance can be obtained from the mother liquors by precipitation with water, and recrystallization from absolute alcohol. Yield about 70 % of the theoretical.

2. *Preparation of the azoyl chloride*

50 g. of the dry acid are mixed with 150 ml. of pure dry benzene. A solution of 40 g. of SOCl<sub>2</sub> in 100 ml. of benzene is then added and the mixture is heated under reflux for 1 hr. on a boiling water bath. The dark red solution is evaporated *in vacuo* and the dry residue redissolved in benzene. The benzene is again evaporated *in vacuo* and the operation repeated a third time. Finally the residue is dissolved in the minimum of boiling light petroleum (b.p. 60–80°), the hot solution is filtered through a heated Jena glass filter and the clear solution slowly cooled to 0°. Bright red crystals are obtained. Recrystallization may also be carried out from boiling benzene. In this case, beautiful dark red crystals with a purple metallic glint are obtained, m.p. 93°. The yield is quantitative. (Found: C, 64.04; H, 3.91; N, 11.8; Cl, 14.33 %. C<sub>13</sub>H<sub>9</sub>ON<sub>2</sub>Cl requires C, 63.8; H, 3.71; N, 11.5; Cl, 14.5 %.)

Recently Ladenburg *et al.* [1938] published a method for the preparation of azobenzene-*p*-benzoyl chloride. These authors state that they had difficulty in preparing the chloride from the acid, but were successful in preparing it by the action of a large excess of boiling thionyl chloride on a mixture of the acid with 2½ times its weight of Na<sub>2</sub>CO<sub>3</sub>. They give m.p. 93–94° for the chloride. During the course of my work I have prepared several hundred g. of the chloride from the acid by the method described in the present paper and have always obtained a quantitative yield.

3. *Esterification of glucose; preparation of α-penta-azoyl-d-glucopyranose*

8.2 g. of well pulverized dry azoyl chloride are added to 50 ml. of pure pyridine (twice distilled over barium oxide) and cooled to –20°. The mixture is shaken for 30 min. at –20° and 1 g. of thoroughly pulverized dry glucose (6 hr. at 80° over P<sub>2</sub>O<sub>5</sub> *in vacuo*) is added. The mixture is kept with frequent shaking for 4 days at 12°. It is then cooled to –20°, and 4 ml. of absolute methanol (dried over barium oxide and twice distilled), are added. The mixture is kept for 3 hr. at –20°, with frequent shaking and then for 12 hr. at 0°. The solid which separates is collected, dissolved in chloroform, precipitated with absolute ethanol, again collected and washed with absolute ethanol. The red powder thus

obtained is recrystallized from dioxane. Red crystals are obtained, m.p. 234–236°. Yield about 80 % of the theoretical. (Found: C, 69.32; H, 4.14; N, 11.36 %.  $C_{71}H_{52}O_{11}N_{10}$  requires C, 69.8; H, 4.29; N, 11.47 %.)  $[\alpha]_{D}^{20} = +193^\circ$  (chloroform;  $c=1$ ).

#### 4. Esterification of fructose

8.2 g. of well powdered azoyl chloride are added to 50 ml. of pure dry pyridine, previously cooled to  $-20^\circ$ ; the mixture is shaken for 30 min. at  $-20^\circ$ , and 1 g. of well powdered dry fructose which had been dried in a very thin layer for 24 hr. over  $P_2O_5$  *in vacuo* is added. The mixture is shaken for 4 hr. at  $-20^\circ$ , and then left for 4 days at  $-12^\circ$ , being frequently shaken during this time. It is then cooled to  $-20^\circ$ , and 4 ml. of absolute methanol added. The mixture is kept for 3 hr. at  $-20^\circ$  and then for 12 hr. at  $0^\circ$ . The solution is filtered and the filtrate evaporated *in vacuo* at room temperature in an apparatus designed by the author.<sup>1</sup> The residue is dissolved in the minimum of hot benzene and precipitated with absolute ethanol. The substance is recrystallized from  $CCl_4$  and is obtained in the form of red crystals, m.p. 135–136°. (Found: C, 69.32; H, 4.47; N, 11.25 %.  $C_{71}H_{52}O_{11}N_{10}$  requires C, 69.8; H, 4.29; N, 11.47 %.)  $[\alpha]_{D}^{20} = -345^\circ$  (chloroform;  $c=1$ ).

#### 5. Chromatographic separation of a mixture of azoyl esters of glucose and fructose

(a) *Preparation of the adsorption column.* 70 g. of silica (pure precip. B.D.H.) are mixed with 300 ml. of a solution of 25 % benzene (pure dry) in dry light petroleum (b.p. 60–80°); the mixture is kept for about 30 min. The adsorption column is prepared with this mixture in the following way: a part of the mixture is poured into a tube 30 mm. in diameter and 400 mm. high. The bottom of the tube is closed by a wad of cotton-wool, on which is placed a perforated disc of porcelain. Establishing a pressure by compressed nitrogen from the top of the tube, the liquid is filtered through the column of silica, until only a few mm. of liquid remain above the surface of the silica. At this stage a fresh quantity of the mixture is poured into the tube and the filtration continued. This is repeated until the column acquires a height of about 300 mm. when a second perforated porcelain disc is placed at the top of the column, and about 100 ml. of the benzene-petroleum mixture (25 : 75) is filtered by pressure through the column, so that the latter is made compact and homogeneous before beginning the chromatographic adsorption. During this operation, as during the entire chromatographic adsorption, the column must never become dry. The pressure of the nitrogen is regulated during the formation of the column so that the liquid filters through the column at a speed of 6–10 ml. per min. A speed of 3–4 ml. per min. is used during the adsorption and development of the chromatogram.

(b) *Adsorption.* In a typical experiment a solution of azoyl esters was prepared in the following way: 200 mg. of the glucose ester and 200 mg. of the fructose ester were dissolved in 40 ml. of chloroform, 80 ml. of benzene and 80 ml. of petroleum are added and the solution was filtered through the column. During the operation the formation of two red layers, separated by an orange layer was observed at the top of the column. When filtration of the solution of esters was nearly com-

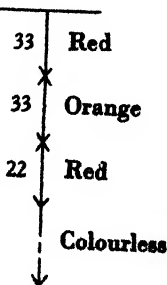


Fig. 1.

<sup>1</sup> The apparatus is now obtainable through Gallenkamp & Co. Ltd.

plete, so that only a few mm. remained above the column, the chromatogram shown in Fig. 1 was observed. The numbers indicate the thickness of the layers in mm.

For the development of the chromatogram, 800–900 ml. of the benzene-petroleum mixture (25:75) were filtered through the column, the current of nitrogen being maintained at the end of the filtration until the column was completely dry. The distribution of the chromatogram at the end of the procedure is shown in Fig. 2.

40	Dark orange	Layer A	After elution 172 mg. of substance				$[\alpha]_{6480}^{20} = -323^{\circ}$ (theor. $-345^{\circ}$ )
65	Nearly colourless	„ x	„	„	17	„ „ „	
2	Orange	„ B	„	„	16	„ „ „	
6	Nearly colourless						
30	Dark orange	„ C	„	„	166	„ „ „	$[\alpha]_{6480}^{20} = +171^{\circ}$ (theor. $+193^{\circ}$ )
105	Nearly colourless	„ y	„	„	9	„ „ „	
2	Orange	„ D	„	„	1	„ „ „	
	Colourless				381 mg. (theor. = 400 mg.)		

Fig. 2.

Fig. 2.

(c) *Elution.* For the separation of the different layers, the tube was held horizontally and, beginning with the upper layer, the column was scraped with a spatula, each layer being taken out separately and placed in a flask. For the elution of the adsorbed substance the powder was suspended in a mixture of methanol and chloroform (2:8), left for 12 hr. at room temperature and treated in the following way. Each mixture was filtered through a Jena glass filter No. G 3 (diameter 25 mm. height 150 mm.), a mixture of methanol and chloroform being drawn slowly through the powder on the filter until this became colourless. The clear filtrate was then evaporated *in vacuo*. The residue was dissolved in a small quantity of  $\text{CCl}_4$ , the solution filtered and the filtrate precipitated with light petroleum (B.P.  $40-60^{\circ}$ ). After collecting the precipitate it was dried *in vacuo*. The yields of the substances eluted in this way are indicated in Fig. 2. One can see from the values of the specific rotation that, after a single adsorption, the substances are nearly pure. A second adsorption, carried out on the individual fractions, leads to the isolation of the pure substances.

Work upon the separation of other sugars and polysaccharides by similar methods is in progress.

#### SUMMARY

1. By the action of azobenzene-*p*-benzoyl chloride (azoyl chloride) on glucose or fructose, in pyridine solution, coloured azoyl esters are obtained.
2. These esters can be adsorbed from their solution in organic solvents by various adsorbents from which they can be recovered by elution.
3. The adsorption is selective, allowing the separation of mixtures of these esters into their components by chromatographic adsorption.
4. The yields of pure substances, recovered from such mixtures are nearly quantitative.

5. The separation of a very small quantity (for example 20 mg.) of the sugar azoyl esters, can be executed by this method.

I wish to take this opportunity of thanking the Governing Body of the Lister Institute, for the hospitality of its laboratories and to Prof. R. Robison for the kindly interest he has shown in this investigation. I am indebted also to Dr Kenyon who placed at my disposal a cadmium lamp for polarimetric measurements.

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# CXXIV. PARATHYROID HORMONE

## I. ASSAY

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COLLIP's original method of assay of parathyroid hormone [Collip & Clark, 1925] presents certain serious disadvantages for routine laboratory use. Not only are dogs expensive and troublesome to maintain, but in practice it is impossible to obtain a sufficiently large group of animals of one breed. As a result, individual differences will be greater in a group of dogs than in an equally numerous group of inbred laboratory animals, such as the mouse and rat colonies available in every biological laboratory. Methods based on prevention of the toxic effects of injections of Na oxalate or  $MgSO_4$  into mice or rats suffer from the disadvantage of requiring extremely numerous groups of animals, in order to give significant results [cf. Burn, 1937]; moreover, individual variations in susceptibility to both the toxic factor and the hormone tend still further to increase the variance. Dyer's rat urine method [1932; 1933; 1935] appeared to afford the most hopeful basis for the elaboration of a practicable and relatively trustworthy method of assay, an attempt at which is described in the present paper.

### EXPERIMENTAL

**Material and methods.** The animal material consisted of male albino rats, of the State Hygiene Institute's inbred Wistar strain, weighing 150–200 g. each. Groups of 5 rats were placed in glass metabolism cages, of a type similar to that used by Dyer [cf. Burn, 1937], but with certain minor improvements. The cage (Fig. 1) consists of an inverted glass bell-jar, with a 0.5 cm. mesh Ni-plated brass wire netting floor and ceiling. The neck of the bell-jar is fitted with a thin glass rod, serving for suspension of a glass bulb, the drawn-out part of which dips into a 100 ml. Erlenmeyer flask, standing in a petri dish.

The dish is covered with a piece of fine-mesh wire gauze, in the form of a truncated cone. The cage, dish and outside of the flask are rinsed with small amounts of hot acetic acid once daily, the washings being added to the urine. The rats are fed once daily in a separate cage, the urine voided during this time being filtered immediately after feeding, and added to the main portion. Distilled water is allowed *ad lib.*

The diet consists of a mixture of wholemeal rye flour 70, evaporated skim milk 10, commercial casein 6, linseed cake 15, dried liver 3,  $CaCO_3$  0.5 and NaCl 0.5 parts, made into a stiff paste with 50 g. of butter and one raw egg per kg. of

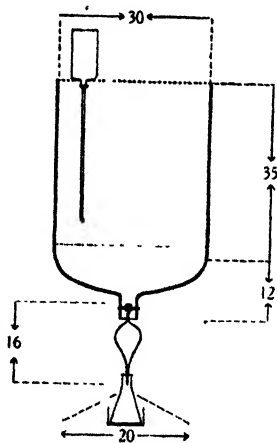


Fig. 1. Metabolism cage  
(to scale).

mixture, together with the necessary amount of hot water. The mass is kneaded into ovoids, each weighing about 15 g., five of which are placed in the feeding cage. Feeding usually lasts about 1 hr., and scattering is practically absent. The combined urine + washings are diluted to 100 ml. in measuring flasks, and Ca is determined by the method of Truszkowski *et al.* [1938]. The values in all cases refer to urinary Ca output per 100 g. of rat per 24 hr.

### RESULTS

The appraisal of the potency of parathyroid preparations by a rat urine method requires that the standard deviation in urinary Ca output be determined for uninjected animals, in order to know what rise in urinary Ca might be regarded as being significant. Typical daily Ca excretion curves are given in Fig. 2,

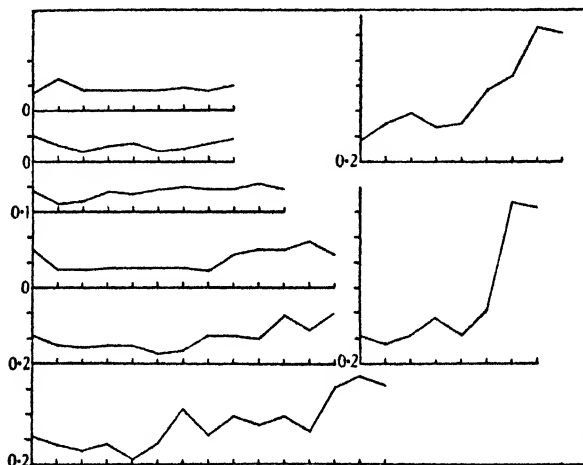


Fig. 2. Daily Ca excretion curves. Ordinates: Ca in 0.1 mg. Abscissae: days.

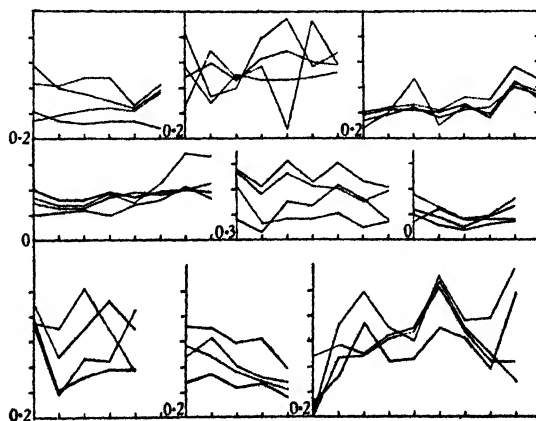


Fig. 3. Daily Ca excretion curves, for four groups studied simultaneously. Ordinates: Ca in 0.1 mg. Abscissae: days.

from which it appears that considerable variations in 24 hr. urinary Ca output may exist (see also Fig. 3), in spite of every precaution to avoid loss or contamination of urine, and of maintenance of standard feeding and living conditions.

Fig. 3 represents a number of groups of curves obtained for four groups of rats studied simultaneously. A general tendency towards parallel fluctuations is evident, suggesting that some factor responsible for variation in urinary Ca output affects all four groups simultaneously; the fluctuations are due not so much to individual group variability as to the action of some external, uncontrolled factor. As to the nature of this factor no basis for speculation is available. The phenomenon described may, however, be of practical importance for assay purposes, in as much as whilst the fluctuations for a given group of rats may be considerable, yet the differences between this group and a second control group may vary within narrower limits.

Fig. 4a represents the distribution of results of 792 determinations of urinary Ca (mg./100 g./24 hr.), under standard conditions, and Fig. 4b the same, for differences (283 values) between test and arbitrarily selected control groups. In

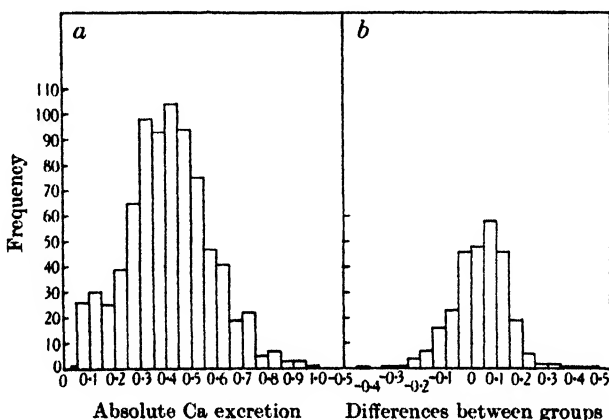


Fig. 4. Distribution of daily Ca excretion for groups of rats.

both cases the results correspond with a normal distribution. The standard deviation was calculated for both groups of results, from the equation

$$\sigma = \lambda \sqrt{\frac{\sum a^2 d}{n} - \left(\frac{\sum ad}{n}\right)^2 - \frac{\lambda^2}{12}},$$

where  $\lambda$  is the class interval (0.05 mg.),  $a$  is the deviation,  $d$  the frequency and  $n$  the number of group-days. On this basis, Ca excretion, in mg./100 g. live wt./24 hr., was found to be 0.404, with  $\sigma = 0.166$ , whilst the difference between one group and another amounted to 0.064, with  $\sigma = 0.066$ . It follows that the standard deviation is 2.5 times as great for absolute Ca excretion as for the differences between groups. In other words, a rise or fall in Ca excretion equal to 0.166 mg./100 g./24 hr. could be expected once in 3 days, and in the difference between two groups on a given day once in 100 days. The conclusion is that a rise in the difference between two groups, of which one had received parathyroid hormone, is a more trustworthy indication of the activity of the hormone than is the absolute Ca rise in one group only.

#### *Effect of injection of parathyroid hormone*

Solutions of parathyroid hormone were prepared by a method based substantially on that of Tweedy [1930], from different batches of ox glands. Owing to technical difficulties, both in procuring large batches of glands, and in work-



ing them up in one operation, our preparations varied considerably in potency. Fig. 5*a* represents the effect of an injection, and Fig. 5*b* shows how the quantitative effect of this injection is evaluated. The curves of Fig. 5*a* represent daily variations in Ca excretion in two groups of rats. An injection of parathyroid hormone was given to one of the groups (curve 2) at the beginning of the 24-hr.

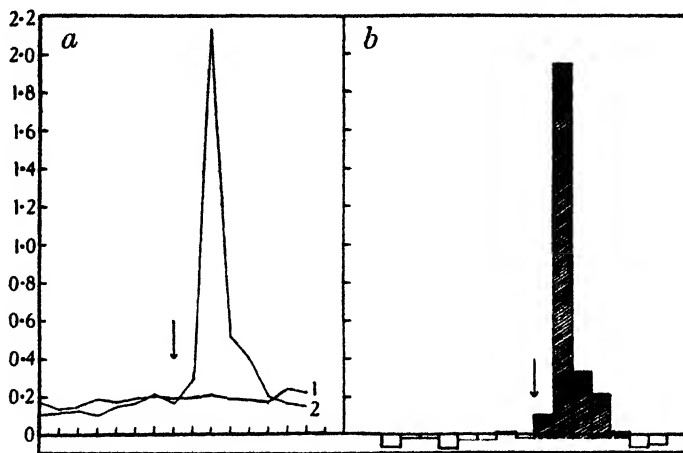


Fig. 5. Effect of injection of parathyroid hormone. Ordinates: Ca in 0.2 mg. Abscissae: days.

period marked by an arrow. The curve then rises and falls steeply, the effect being completed within 72 hr. The histogram (Fig. 5*b*) consists of a series of columns, each being the difference between the values of curves 1 and 2 on a given day; these differences may be positive or negative. The response to the injection is evaluated as being the sum of the areas of the columns (shaded) above the level of the pre-injection column. This procedure involves the arbitrary assumption that the level on the day preceding injection remains constant over the test period; actually, since the standard deviation is 0.066, it may vary considerably. The response is, however, considerably greater than these possible variations, which should not involve an error  $> 10\%$ ; such a margin of error is generally regarded as permissible in biological assay methods. The original histograms are drawn on millimetre square paper, to a scale of 1 cm./0.1 mg. Ca and per 24 hr. period, so that the sum  $S$  is expressed in sq. cm. We would propose that with this method of plotting 1 sq. cm. ( $= 0.1$  mg. Ca) be tentatively taken as being equivalent to 1 rat unit of parathyroid activity, pending the production and adoption of a reference standard. The potency of solid preparations of parathyroid hormone would then be best represented as  $S/\log D$ , where  $D$  is the dose in mg.

The groups of rats were discarded after each experiment, in view of the possibility of development of immunity in previously injected animals.

#### *Uniformity of response of different groups of rats to an identical dose of hormone*

An injection of 11.26 mg./100 g. of prep. A<sub>c</sub> was given simultaneously to three groups of rats. The histograms representing the effect are shown in Fig. 6*a*. Fig. 6*b* illustrates the result of a similar experiment, taking 19 mg./100 g. of prep. Y. The values of  $S$  for prep. A<sub>c</sub> at the given dosage level were  $S_1 = 1.3$ ,

$S_2=2.4$ ,  $S_3=2.3$ ; for prep. Y the values were  $S_1=22.6$ ,  $S_2=25.2$ ,  $S_3=22.9$  sq. cm. It follows that the responses differ considerably qualitatively, those to prep. A<sub>c</sub> being distributed over 2, 3 and 1 days respectively for groups 1, 2 and 3, or

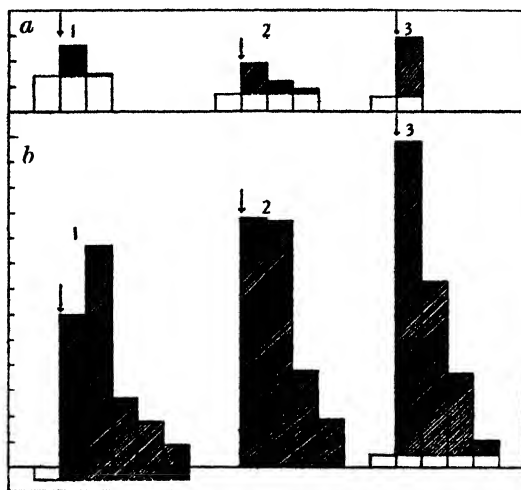


Fig. 6. Effect of equal doses of hormone prep. A<sub>c</sub> (Fig. 6a) and Y (Fig. 6b) given to three groups of rats. Ordinates: Ca in 0.1 mg. Abscissae: days.

rising gradually to a low maximum for group 1 (prep. Y), and abruptly to higher ones in groups 2 and 3. The maximum level is maintained for 48 hr. in group 2, and for 24 hr. only in groups 1 and 3. Return to pre-injection levels is most gradual in group 3, less so in group 2 and least so in group 1. Yet the values of  $S$  differ relatively little for identical doses.

#### *Gradation of response to various doses of a given preparation*

It might be expected that the magnitude of  $S$  should bear some simple relation to the logarithm of the dose, were the assay method described to give a

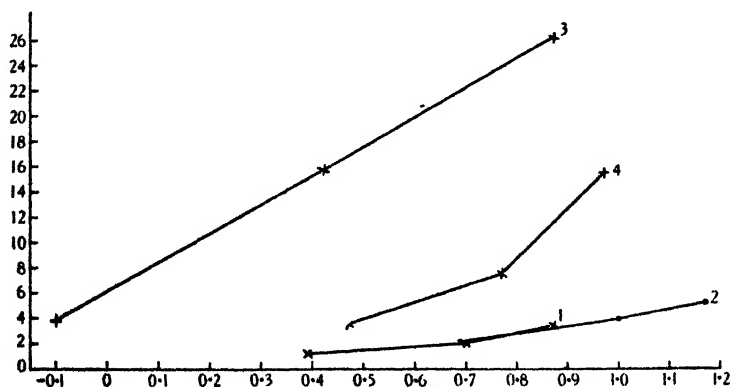


Fig. 7. Relation of dose of hormone to response. Ordinates: Response  $S$ . Abscissae: Logarithm of dose in mg.

true representation of the potency of the hormone preparations. The results obtained are shown in Fig. 7, in which curves 1 and 2 represent the effect of

injections of 2.5, 5.0 and 7.55, and of 5, 10 and 15 mg. of prep. N<sub>a</sub>/100 g. of rat, made at different times into three groups of rats. Curve 2 is a straight line, and two of the points of curve 1 fall on the same line, the lowest dosage giving an aberrant effect. This is due probably to the magnification of the experimental error at this low dosage level. Curve 3 is a straight line joining the points obtained with doses of 0.78, 2.68 and 7.55 mg. of the more active prep. D. Curve 4 is for doses of 2.97, 5.95 and 8.87 mg. of prep. Z, and deviates to some extent from the rectilinear.

On the whole, the results confirm the validity of the assay method proposed.

#### *Effects of multiple or single injections*

It is known that for a number of hormones a better response is obtained when the dose is divided into a number of smaller ones, administered at intervals, and Dyer [1933] has reported the same for parathyroid hormone. A test made with one of our preparations showed that  $S$  was 20.1 when a given dose was administered in two injections on 1 day, and 26.0 when the same dose was spaced over 3 days. Whilst this result confirms Dyer's findings, yet the advantage does not in our opinion counterbalance the inconvenience of multiple injections, and of the prolongation of the test period. In subsequent work the hormone was administered in two injections at an interval of 4 hr.

#### *Units*

The present units are the Collip dog unit (one-hundredth of the amount of hormone causing a 5 mg. rise in dog serum Ca, after 15 hr.), or the Hanson unit, being one-hundredth of the amount of hormone raising the serum Ca of a parathyroidectomized dog by 1 mg. within 6 hr. These units are not very satisfactory, but no better ones have been proposed. Dyer bases his assays on comparison with the declared potency of a commercial preparation; such a procedure presents obvious disadvantages. The most satisfactory methods are those based on comparison with a standard preparation, but no such preparation is available in the present case. A sample of hormone was prepared in this laboratory about a year ago, and its stability is now being studied, with a view to the ultimate adoption of this or a similar preparation as a reference standard. In the meantime, the assay method proposed can be applied, if possible taking some reliable commercial preparation for comparison. In a test made in our laboratory it was found that a dose of 10 Collip units/100 g. of rat gave a response of  $S = 19.3$ , whence it would follow that 1 Collip unit is equivalent to about 2 of our rat units. The most potent preparation so far obtained in our laboratory had an activity of about 6 rat units/mg.

#### SUMMARY

1. Fairly considerable fluctuations in urinary Ca output are established for groups of rats kept under standardized conditions.
2. A tendency towards parallel variation in Ca output of four different groups during given periods of time is evident; the standard deviation of differences between the various groups is 0.064 mg./100 g. live wt./day (283 values), as compared with 0.166 mg. for absolute urinary Ca output (792 values, of which the mean is 0.404 mg.).
3. The response to various doses of a given preparation of parathyroid hormone is proportional to the logarithm of the dose. For a given dose approximately the same response is given by different groups.

4. A graphical method of evaluating the potency of parathyroid preparations, based on that of Dyer, is described. Pending the establishment of a reference standard, it is proposed that activity be expressed in terms of excess of urinary Ca excretion following injection.

5. The proposed rat unit is defined as being one-tenth of the amount of hormone giving a total rise in urinary Ca output of 1 mg.

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# CXXV. FORMIC DEHYDROGENASE OF *BACTERIUM COLI*: ITS INACTIVATION BY OXYGEN AND ITS PROTECTION IN THE BACTERIAL CELL

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*(Received 19 April 1939)*

QUASTEL & WHETHAM [1925] showed that washed suspensions of *Bact. coli* will reduce methylene blue in the presence of formate and that the rate of reduction under standard conditions is greater with formate as substrate than for any other substance, with the exception of the sugars. Stickland [1929] showed that if a suspension of the organism is incubated with *liquor pancreaticus* then the activity of the formic dehydrogenase with methylene blue increases until it reaches 4 or 5 times its original value after several days. The activity of other dehydrogenases also increases at first but then rapidly decreases until the preparation is eventually active only with formate. After filtration of this preparation through a glass filter, 50–60 % of the activity is found to reside in the filtrate which contains large quantities of cell debris. When these are removed by high speed centrifuging, the supernatant fluid is almost inactive. Although the original bacterial suspension is able to oxidize formate to completion, the dehydrogenase preparation is unable to react with  $O_2$  in the presence of formate. A similar phenomenon was encountered by Stephenson [1928] with a preparation of lactic dehydrogenase from *Bact. coli* but, in that case, the preparation was able to utilize  $O_2$  as H-acceptor in the presence of a trace of methylene blue as carrier. Stickland was unable to obtain an  $O_2$  uptake with his preparation by the addition of 0.5 ml. methylene blue (1/5000) but obtained, with or without the dye, a small uptake amounting to 20–30  $\mu$ l. of  $O_2$ . He was unable to find any explanation of this effect and could only obtain complete oxidation of formate by, first, the anaerobic reduction of a large quantity of methylene blue by the formate in the presence of the preparation, followed by the aerobic re-oxidation of the reduced methylene blue.

In the course of studies of the enzyme systems of *Bact. coli* by investigation of the material obtained by crushing cell suspensions in the wet-crushing mill of Booth & Green [1938] it was decided to reinvestigate this problem, with the results set out in the following paper.

*Technique.* The organisms in all cases were grown on the surface of broth-agar in Roux bottles for 24 hr. They were then washed off with distilled water, spun out, washed twice in water and finally suspended in distilled water. The strength of such suspensions was determined by means of a photoelectric turbidimeter [Clifton *et al.* 1935]. The  $Q_{O_2}$  ( $\mu$ l.  $O_2$  taken up/hr./mg. dry weight of organism) was determined in Warburg manometers; 1 ml. each of the bacterial suspension,  $M/40$  Na formate and  $M/10$  phosphate buffer at pH 6.0 being placed in the main compartment of the manometer cup and 0.2 ml. 10 % NaOH in the centre cup. All values of  $Q_{O_2}$  are corrected for the blank respiration of the organism. The activity with methylene blue was determined in Thunberg tubes evacuated and incubated at 37°. The following quantities were used: 1 ml. bacterial suspension, 1 ml.  $M/10$  Na formate (in the hollow stopper) and 1 ml.

phosphate buffer at pH 6.0, in the presence of 0.2 ml. of 0.5 % methylene blue. The substrate was tipped into the reaction mixture after equilibration and the time ( $T$  sec.) taken to reduce the dye to completion was noted. The activity of the dehydrogenase towards methylene blue is given as  $Q_{MB}$ , defined as:  $Q_{MB} = \mu\text{l. O}_2$  equivalent to the methylene blue reduced/hr./mg. dry weight of preparation. Under the experimental conditions described, this is equal to  $108,000/TW$ , where  $W$  = dry weight of preparation.

For the preparation of cell-free material from the organisms, 30–40 Roux bottles were inoculated with the organism and incubated for 24 hr. The total quantity of cells so obtained was suspended, after washing, in 50 ml. of water and then ground for  $2\frac{1}{2}$  hr. in the Booth-Green mill. The cream obtained in this manner was investigated for formic dehydrogenase activity as below.

*Distribution of the formic dehydrogenase in the crushed material*

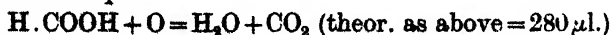
The activity of the ground material as formic dehydrogenase was investigated by the methylene blue technique and the results compared with the activity of the original washed suspension of organisms. Next, the ground material (1) was centrifuged for 30 min. at 3000 r.p.m.; this treatment provides two fractions: a brownish-yellow opaque supernatant fluid (2) and a thick sediment (5). The material (2) was then further centrifuged at 11,000 r.p.m. for 20 min. and gave a clear yellow fluid (3). Finally, this clear fluid was filtered through a Seitz filter, giving a water-clear fluid (4). The dry weight of each fraction was determined by drying suitable amounts in tared vessels to a constant weight in a steam oven and the methylene blue activity ( $Q_{MB}$ ) then determined as above. The results are set out in Table I.

Table I. *Distribution of formic dehydrogenase in crushed material*

Material	$Q_{MB}$	
	Exp. 1	Exp. 2
Washed suspension of organism	183	118
Ground material (1)	104	72
Opaque supernatant fluid (2)	38	25
Clear yellow fluid (3)	13	9
Filtered fluid (4)	0	0
Washed sediment (5)	218	136
Dried sediment	45	11

It is obvious from these results that the formic dehydrogenase activity resides in the solid particles, as fractional removal of these leads to a steady loss of activity, the final filtered fluid being inactive. Accordingly further work was carried out on the washed sediment (1) only. Attempts to remove the enzyme from the surface of the solid material by (i) extraction with phosphate buffers of pH 5–10 either before or after digestion with trypsin, (ii) digestion with trypsin, (iii) digestion with papain etc. were completely unsuccessful.

*Oxidation of formate by the sediment.* Reference to Table II shows that the sediment gives a  $Q_{O_2}$  of approximately the same value as that obtained with the whole organism but its  $Q_{MB}$  has decreased below that for the organism. The sediment incubated under the usual conditions with 1 ml. of  $M/40$  formate gives a total oxygen uptake of  $288 \mu\text{l.}$  so that the oxidation is taken to completion in agreement with the equation:



This reaction has been established for the whole organism by Cook & Stephenson [1928].

Fig. 1 shows the effect of  $pH$  on the  $Q_{MB}$  and the  $Q_{O_2}$ . The former is more sensitive to changes in  $pH$  than the oxidation system. A working  $pH$  of 6.0 has been adopted for all experiments.

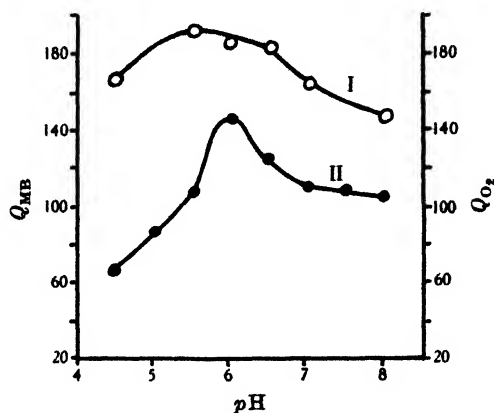


Fig. 1. Effect of  $pH$  on  $Q_{O_2}$  (I) and  $Q_{MB}$  (II) with formic dehydrogenase of the sediment.

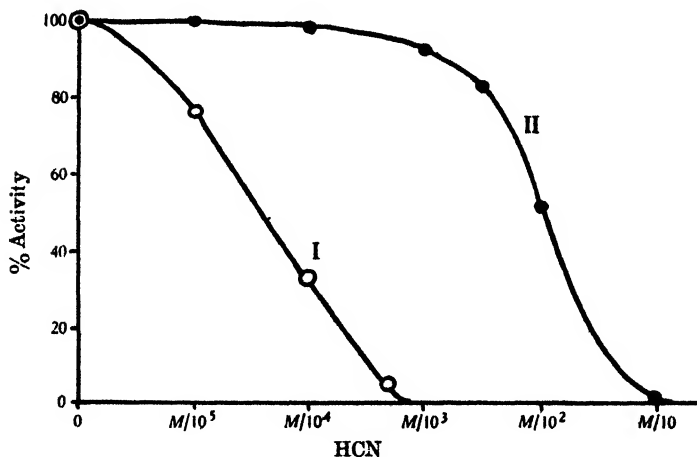


Fig. 2. Effect of HCN on  $Q_{O_2}$  (I) and  $Q_{MB}$  (II) of formic dehydrogenase in sediment.

Fig. 2 shows the effect of cyanide on the  $Q_{MB}$  and  $Q_{O_2}$ . The  $O_2$  uptake is completely inhibited by a concentration of  $M/1000$  cyanide but this concentration inhibits the methylene blue activity by 8 %, complete inhibition of the dehydrogenase only being obtained with  $M/20$  cyanide. During the  $Q_{O_2}$  determinations mixtures of alkali and cyanide were used in the centre cup according to the technique of Krebs [1935]. From these results it appears that formic dehydrogenase differs from most dehydrogenases in being inhibited by cyanide. The preparation inactivated in the presence of  $M/10$  cyanide is not restored by washing three times in 1000 times its weight of water so that the inhibition would appear to be irreversible. Adler & Svreenivasaya [1937] have also shown that formic dehydrogenase extracted from peas is inhibited by cyanide.

*Effect of digestion of the sediment with trypsin.* The sediment was next incubated in the presence of phosphate buffer at pH 7.5 with trypsin. 1 % fluoride was used as antiseptic as this has no effect upon the dehydrogenase activity. The trypsin preparation used was the "Pangestin" preparation of "Difco", in a concentration of 10 % of the weight of material to be digested. At intervals samples were withdrawn, washed thoroughly until free from trypsin, the dry weight determined and the  $Q_{O_2}$  and  $Q_{MB}$  investigated. Also the total  $O_2$  uptake obtained in the presence of 1 ml.  $M/40$  formate (theory =  $280 \mu l.$ ) with an amount of material equivalent to 2 mg. of the original sediment was determined. The results are given in Table II.

Table II. *Effect of digestion of sediment with trypsin*

Material	Digestion time	$Q_{O_2}$ formate	$Q_{MB}$	Total $O_2$ uptake ( $\mu l.$ )
Washed suspension of organism	—	171	158	?
Washed sediment	—	166	107	290
	18 hr.	152	226	156
	40 hr.	132	366	102
	60 hr.	118	334	72
	6 days	114	603	34
	7 days	35	475	15
	8 days	—	442	0

From these figures it is seen that:

- (1) The initial  $Q_{O_2}$  falls steadily but slowly for 6 days and then falls rapidly to zero by the 8th day.
- (2) The  $Q_{MB}$  rises steadily until the 6th day and then falls but has 300–400 % of the original activity when  $Q_{O_2} = 0$ .
- (3) The total  $O_2$  consumption decreases rapidly as digestion proceeds.

During this digestion the dry weight of the sediment decreases to 25 % of its original value and the rise in  $Q_{MB}$  is largely accounted for by the decrease in dry weight due to removal of inactive material. However, in some cases, an increase in actual dehydrogenase activity amounting to some 200 % has been observed—as found by Stickland [1929]. Further digestion after the 8th day in the presence of fresh trypsin leads to a slow but steady loss in dehydrogenase activity.

Sediment allowed to autolyse in the presence of fluoride at  $37^\circ$  showed the changes set out in Table III. The experiments were all carried out with an amount of material equivalent to 2 mg. of the original sediment and the results show an increase in  $Q_{MB}$  while the  $Q_{O_2}$  remains unchanged but the total oxygen uptake is limited to 100–150  $\mu l.$  after 6 days' autolysis.

Table III. *Effect of autolysis of sediment*

Material	Autolysis time	$Q_{O_2}$ formate	$Q_{MB}$	Total $O_2$ uptake ( $\mu l.$ )
Washed suspension of organism	—	171	158	?
Washed sediment	—	166	107	290
	20 hr.	210	281	174
	40 hr.	184	281	154
	5 days	182	356	141
	6 days	153	324	137

It will be convenient to refer, in the following experiments, to the preparations as follows:

Material A. The untreated, washed sediment.



**Material B.** The sediment after 7 days' autolysis, able to oxidise 1 ml.  $M/40$  formate to 50 % completion.

**Material C.** The sediment after 7 days' digestion, with a high  $Q_{MB}$  but unable to react with  $O_2$ .

In all cases a quantity of material, in suspension, was used equivalent to 2 mg. dry weight of material A. The materials were kept in water suspension in the presence of 1 % fluoride in the ice-chest; for use they were spun out, washed and resuspended in water. Desiccation of the materials leads to a marked loss of activity, see Table I.

It appears from the results obtained that the material C contains the formic dehydrogenase but that this is unable to react with  $O_2$  in the absence of some factor which has been removed by the digestion with trypsin. This would also explain the differences between the methylene blue and  $O_2$  activities in Figs. 1 and 2.

*Spectroscopic examination of the materials.* A sample of material A containing 50 mg./ml. was examined under the microspectroscope. No absorption bands were visible and slight warming produced no alteration. When hydrosulphite was added, the bands of cytochrome *b* were easily visible. On shaking the material with air the bands disappeared again. Next, formate was added to a fresh sample and, after momentary warming, the bands appeared strongly. The addition of  $M/10,000$  cyanide had no effect on either the reduction of the cytochrome by formate or the reoxidation by shaking with air.  $M/50$  cyanide inhibited the reduction of the cytochrome in the presence of the formate but not by hydrosulphite. This shows, from Fig. 2, that the formic dehydrogenase of the sediment can react with  $O_2$  through the cytochrome system.

Examination of a similar sample of material B gave similar results except that the appearance of the cytochrome bands was slower in this case when the material was reduced with formate.

Material C, on the other hand, appeared to contain little or no cytochrome as no absorption bands appeared either on warming with formate or reduction with hydrosulphite. When a preparation of cytochrome *c*, extracted from heart muscle, was added to material C in the presence of formate and warmed, the spectrum of reduced cytochrome *c* appeared and the bands remained until a preparation of cytochrome oxidase from heart muscle was added, when the bands disappeared on shaking with air. Thus it would appear that the cytochrome *c*—cytochrome oxidase system can act as a carrier system in conjunction with the formic dehydrogenase of material C.

However, when material C was shaken in a Warburg manometer with phosphate buffer, formate and 0.2 ml. of a 0.06 % cytochrome *c* preparation and cytochrome oxidase, no significant  $O_2$  uptake could be obtained. Further, material B, which contains cytochrome, is unable to oxidize 1 ml. of  $M/40$  formate to completion but gives an  $O_2$  rate which falls off steadily to zero after about an hour. The effect appears to be due to inactivation of the enzyme system and this was the next effect studied.

*Inactivation of material B.* Fig. 3 shows the oxygen consumption under standard conditions with varying initial concentrations of formate and 1 mg. (dry weight) of material B. Table IV shows the percentage theoretical uptake obtained in each case together with the initial  $Q_{O_2}$  determined for the first 10 min. of the reaction. The higher the initial formate concentration, the higher the initial  $Q_{O_2}$  but the lower the percentage oxidation of substrate.

Stackland [1929] stated that the possible by-products of the dehydrogenation, oxalic acid and formaldehyde, do not affect the reaction until their concentration

reaches a higher level than could be formed from the formate present. This was confirmed,  $M/100$  oxalate or  $M/100$  H.CHO having no effect.

Particular attention was paid to the possibility of the formation of  $H_2O_2$  and this was ruled out from the following considerations:<sup>1</sup>

(1) All the preparations contain powerful catalase activity.

(2) The addition of alcohol in an attempt to obtain a coupled oxidation in the presence of catalase [Keilin & Hartree, 1936] has no effect on the  $O_2$  consumption. Further, tests for acetaldehyde after such experiments proved negative. The test used was the fuchsin sulphurous acid spot test which, according to Feigl [1937], will detect  $4\mu g.$  of acetaldehyde.

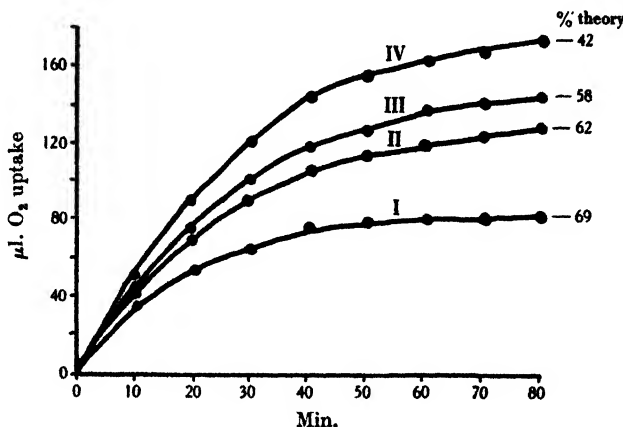


Fig. 3. Effect of initial formate concentration on inactivation, see Table IV. Initial concentration: I,  $M/240$  formate. II,  $M/160$  formate. III,  $M/120$  formate. IV,  $M/80$  formate.

Table IV. *Effect of initial formate concentration on inactivation*

ml. of $M/40$ formate added	Theoretical $O_2$ uptake $\mu l.$	Actual $O_2$ uptake $\mu l.$	% uptake	Initial $Q_{O_2}$
0.5	140	96	69	142
0.75	210	131	62	148
1.0	280	165	58	208
0.75 ( $M/20$ )	420	175	42	279

(3) No colour can be obtained, after a reaction, with guaiacum and peroxidase.

(4)  $M/3000$   $H_2O_2$ , easily detectable by the above test, has no effect on the  $Q_{O_2}$  or on the total  $O_2$  consumption.

(5) The presence of peroxidase and *p*-phenylenediamine during the reaction has no effect on the  $Q_{O_2}$  or on the total  $O_2$  consumption.

(6) In (5) slight browning of the reaction mixture occurred during the experiment but there was no difference in the depth or rate of appearance of the colour in the experiment and in the control without formate.

In case the effect should be due to a loss or deficiency of some factor in the oxidation carrier system, the addition of the following substances was tested: cozymase, lactoflavin, cytochrome oxidase, cytochrome *c* (0.2 ml. of 0.06 % solution, see p. 1023), boiled organism, malic and fumaric acids. The inactivation was not altered or checked by any of these additions or by combinations of these substances.

<sup>1</sup> See also Bhagvat & Hill (in preparation).

*Conditions for inactivation.* Fig. 4 shows the  $O_2$  uptakes in two manometers containing material B in the usual amount. In manometer 1 (curve 1) 0.5 ml. of  $M/40$  formate was tipped into the reaction mixture at zero time and the reaction allowed to proceed until the preparation was inactivated after 70 min. Then a further 0.5 ml. of  $M/40$  formate was tipped in: the preparation was then unable to oxidize the freshly added substrate. In manometer 2 (curve 2) no formate was added at first but the enzyme preparation was shaken with buffer in air under similar conditions to those in manometer 1. Then 0.5 ml. of  $M/40$  formate was added at the same time as the second addition to manometer 1. In this case the enzyme was able to oxidize the formate readily.

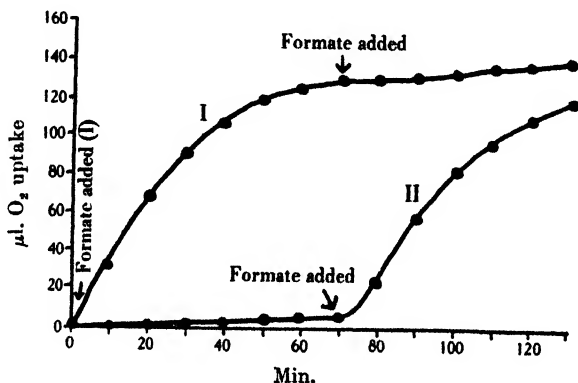


Fig. 4. Effect of presence of substrate on inactivation, see text.

This experiment shows that the enzyme is not inactivated by shaking in air under the experimental conditions but that the presence of the substrate is essential for the inactivation to occur. It also disposes of the supposition that the falling off in the rate of oxidation might be due to the establishment of an equilibrium since, in manometer 1, the addition of further reactant has no effect upon the course of the reaction.

In this experiment, the  $Q_{MB}$  of the preparation at the beginning of the experiment was 475. After shaking in air alone at  $37^\circ$  for 70 min., the value of the  $Q_{MB}$  was unchanged but the enzyme which had been incubated aerobically with the formate for the same time was completely inactivated. Thus the inactivation applies not only to the  $O_2$  uptake but also to the methylene blue activity so that it is the dehydrogenase itself which is inactivated.

In order to determine whether the enzyme would be inactivated anaerobically in the presence of its substrate, 1 ml. of preparation containing 1 mg. dry weight of material B was incubated in a Thunberg tube with 1 ml. of  $M/20$  formate and 1 ml. of phosphate buffer at pH 6; 2 ml. of 0.5 % methylene blue were tipped in slowly from the hollow stopper as the reduction progressed in the evacuated tube. The whole of the dye was reduced, being equivalent to an  $O_2$  consumption of  $300 \mu l.$ , and the  $Q_{MB}$  of the material was unchanged. A similar experiment carried out aerobically in the absence of methylene blue gave an oxygen consumption of  $113 \mu l.$ , after which the enzyme was completely inactivated, having a  $Q_{MB}$  of less than 10.

Thus the dehydrogenase is inactivated only if it is incubated aerobically in the presence of its substrate.

*Effect of incubation with glutathione (GSSG).* Hopkins & Morgan [1938] have shown that the activity of succinic dehydrogenase of animal tissues is completely abolished by incubation with GSSG. The inactive material can then be reactivated by incubation with GSH and the authors suggest that the activity of the enzyme may depend upon the presence of certain SH groups. In order to test whether any such effect could be obtained with the formic dehydrogenase preparation, the following experiment was carried out: a series of Thunberg tubes were made up as set out in Table V and then incubated anaerobically for the times indicated. After this period the material was centrifuged out, washed, resuspended in water and the reduction time determined as usual in each case. It is seen from Table V that GSSG has no effect on the activity in the absence of formate but produces a slow decrease of activity when incubated with the dehydrogenase in the presence of its substrate.

Table V. *Effect of GSSG on formic dehydrogenase*

Tubes ...	Preliminary incubation.		ml. of additions			
	1	2	3	3a	4	4a
Material B (2 mg./ml.)	2	2	2	2	2	2
M/20 phosphate	2	2	2	2	2	2
M/10 formate	—	1	—	—	1	1
M/50 GSSG	—	—	1	—	1	—
M/10 GSSG	—	—	—	1	—	1
Water	2	1	1	1	—	—
Activity after incubation						
Reduction times after						
Material from tube no.	2 hr. incubation		1 hr. incubation			
	min.	sec.	min. sec.			
1	7	0	6 30			
2	7	10	6 30			
3	7	5	6 30			
4	8	5	8 30			
3a	—	—	6 30			
4a	—	—	10 30			

*Reactivation of inactivated enzyme.* 10 ml. of a suspension of material B were incubated in the presence of 10 ml. M/10 formate and 10 ml. M/20 phosphate buffer at pH 6 for 2 hr., being bubbled vigorously with air throughout the incubation period. At the end of that time, a sample was taken and its  $Q_{MB}$  determined. The remainder of the material was centrifuged out, washed once and made up to 9 ml. with water. 1 ml. was placed in each of eight Thunberg tubes and the various additions indicated in Table VI then made. The tubes were incubated, 1 and 3 aerobically and the rest anaerobically, for 1 hr. In the hollow stoppers 1 ml. M/10 formate and 0.2 ml. 0.5 % methylene blue (the latter omitted in tubes 3 and 4) were placed and after the preliminary incubation, the contents of the stoppers were tipped in, all the tubes evacuated and the  $Q_{MB}$  determined in each case. From Table VI it is seen that anaerobic conditions in the presence or absence of formate succeed in reactivating the enzyme. GSH has no significant effect.

Fig. 5 shows the results of an experiment in which the rate of reactivation of a partially inactivated preparation was studied under anaerobic conditions and in the presence of formate anaerobically, by means of an experiment similar to the last in which the contents of the stoppers were added at 30 min. intervals. The presence of the formate accelerates the reactivation process. Since the inactivation process occurs only in the presence of formate, it is possible that the

Table VI. *Reactivation of inactivated dehydrogenase*

		$Q_{MB}$ after treatment
	Original material	442
	After aerobic incubation with formate	43
	Conditions during preliminary incubation	
	Additions	
Tube 1	1 ml. water (aerobic)	43
2	1 ml. water (anaerobic)	366
3	1 ml. water	43
	0.2 ml. 0.5 % MB (aerobic)	
4	1 ml. water	345
	0.2 ml. 0.5 % MB (anaerobic)	
5	1 ml. <i>M</i> /100 GSH (anaerobic)	172
6	1 ml. <i>M</i> /100 GSSG (anaerobic)	108
7	1 ml. boiled organism (anaerobic)	21
8	1 ml. <i>M</i> /10 formate (anaerobic)	377

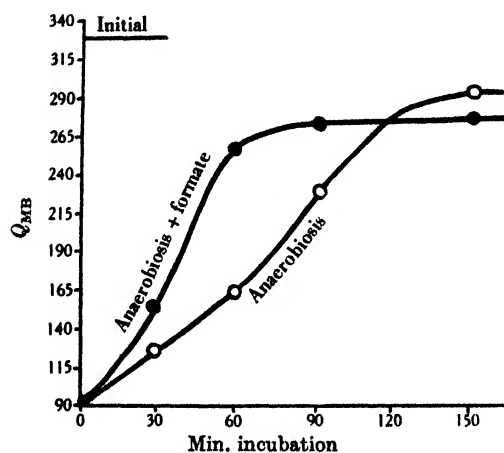


Fig. 5. Reaction of partially-inactivated enzyme by incubating, (a) anaerobically; (b) anaerobically with formate.

effect of anaerobic conditions alone in this last result may be due to the presence of unremoved traces of formate. It has not been possible to establish this as prolonged washing of the inactivated material renders it permanently inactive.

It would seem likely that the reactivation process is one of reduction. Moreover, the conditions necessary for the inactivation of the enzyme in the first place make it appear that the inactivation is associated with an oxidation of the dehydrogenase during its action with its substrate. Reference to Table II shows that the enzyme becomes more susceptible to the inactivation process as digestion of the sediment progresses. Further, it can be shown that the cytochrome content of the sediment decreases during digestion until material C is devoid of cytochrome. The question then arises as to whether the cytochrome normally "protects" the enzyme from inactivation by oxidation and this point will now be considered.

#### *Effect of $O_2$ tension on the inactivation of material B*

A series of manometers were set up with the usual contents for the investigation of the  $Q_{O_2}$  of material B. These were then gassed with  $O_2$ - $N_2$  mixtures containing the following percentages of  $O_2$ : 100, 50, 20, 10, 5, 2½. Fig. 6 shows the  $O_2$

uptakes followed over 10 min. intervals. In pure oxygen, the enzyme is quickly inactivated, the oxygen uptake being reduced to zero in 30 min. As the percentage of  $O_2$  in the gas phase decreases so does the rate of inactivation until, in  $2\frac{1}{2}\%$   $O_2$ , the  $O_2$  consumption is linear for at least 2 hr.

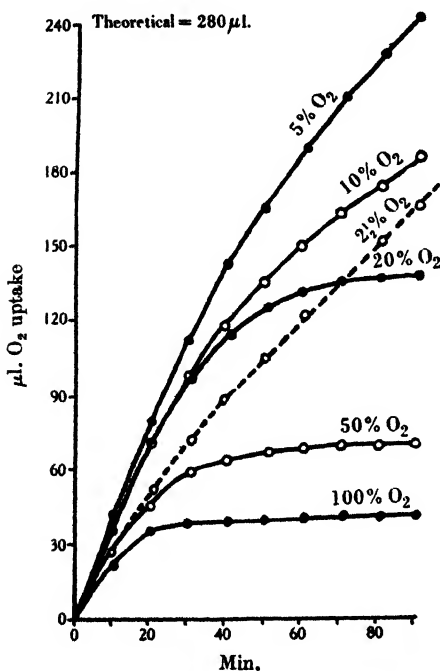


Fig. 6.

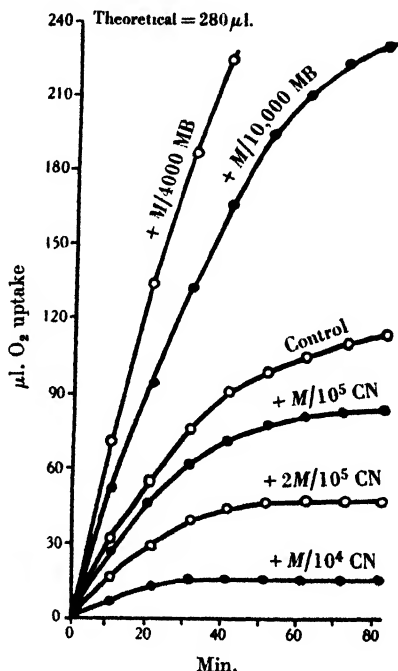


Fig. 7.

Fig. 6. Effect of oxygen tension on inactivation of formic dehydrogenase in material B.

Fig. 7. Effect of (a) MB; (b) CN on inactivation of formic dehydrogenase of material B in air.

Next the effect of altering the amount of potential oxygen-carriers in the system was tried. The rate of reaction of the dehydrogenase with  $O_2$  through carrier systems can be increased by the addition of methylene blue, or decreased, if the natural carrier present is cytochrome as indicated by the spectroscopic observations, by the action of cyanide. Fig. 7 shows the effect of adding methylene blue to final concentrations of  $M/4200$  and  $M/10,500$  and of cyanide to  $M/10^5$ ,  $2M/10^5$  and  $M/10^4$ , the gas phase in all cases being air. Increasing the velocity of the reaction of the dehydrogenase with  $O_2$  through carrier systems results in an inhibition of the inactivation process; while the action of cyanide, which inhibits the reaction through the cytochrome system, speeds up the inactivation of the dehydrogenase.

These effects can be reconstructed using methylene blue as the sole carrier with material C which contains no natural carrier. The material C when shaken with formate aerobically gives an  $O_2$  uptake of 5–12  $\mu$ l. and is then inactivated towards either  $O_2$  or methylene blue. Fig. 8 shows the effect of adding various concentrations of MB to material C and then shaking with formate in air. Low concentrations of the dye—less than  $M/10^5$ —have very little effect but higher concentrations enable  $O_2$  to be utilized to an extent depending upon the

dye concentration: the higher this concentration, the slower the inactivation of the dehydrogenase.

Fig. 9 shows the course of the  $O_2$  uptake by material C in the presence of formate and a constant amount of MB,  $M/4200$ , but with various  $O_2$  tensions. High percentages of  $O_2$  in the gas phase lead to a rapid inactivation of the dehydrogenase while if the  $O_2$  tension is sufficiently reduced, then a linear uptake is obtained over the experimental period. The general picture is thus similar to that of Fig. 6 in which the carrier system is cytochrome.

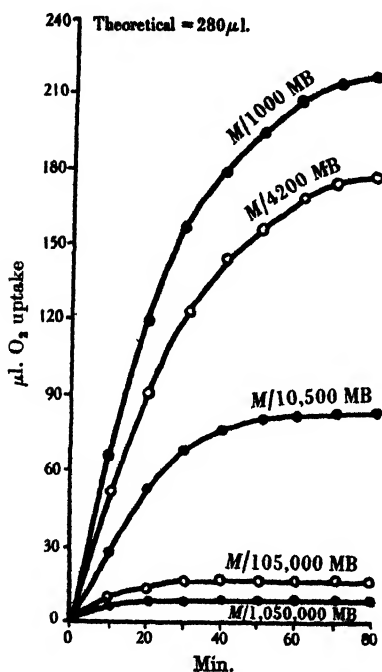


Fig. 8.

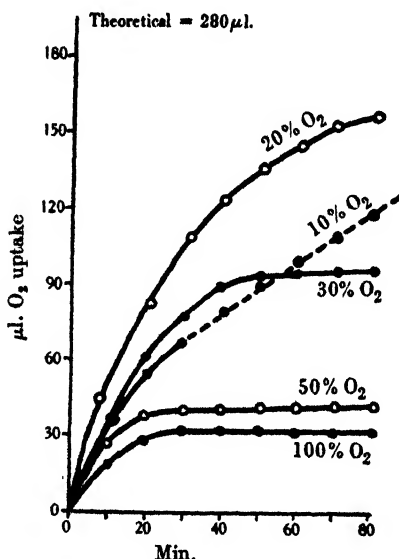


Fig. 9.

Fig. 8. Oxygen uptake with material C in presence of formate and varying concentrations of MB in air.

Fig. 9. Course of  $O_2$  uptake with material C in presence of formate,  $M/4200$  methylene blue and varying oxygen tensions.

In every case, the rate of inactivation of the dehydrogenase is accelerated by decreasing the concentration of the oxygen-carrying systems present, but is inhibited by increasing their concentration. In the presence of a constant amount of carrier, then the rate of inactivation is dependent upon the  $O_2$  tension; the higher the tension, the more rapid the inactivation process.

It would appear that the enzyme can react with  $O_2$  along two paths—directly, leading to inactivation of the dehydrogenase activity, and indirectly through a carrier system; which process predominates depends upon (a) the  $O_2$  tension and (b) the amount of carrier present. Increasing the  $O_2$  tension, while the amount of carrier present is unchanged, would speed up the direct reaction and thus the inactivation process; while increasing the amount of carrier favours the indirect reaction and so diverts the  $O_2$  from the inactivation process. Under physiological conditions it is probable that the reaction through the natural

carrier system is so rapid that the  $O_2$  is diverted from the direct reaction and inactivation proceeds only slowly. This is dealt with later.

**Reconstruction of the cytochrome system.** Previously it has been impossible to obtain an  $O_2$  uptake by material C in the presence of formate by the addition of cytochrome *c* and cytochrome oxidase. It would appear that this result may be due to (a) insufficiently high concentration of cytochrome and (b) too high  $O_2$  tension. Also it must be remembered that cytochrome *c* is not the natural cytochrome involved but no method of preparing the cytochrome *b* of bacteria is yet available. Hence manometers were set up with the following additions:

(1) and (3) 0.5 ml. 1 % cytochrome *c*; 0.3 ml. cytochrome oxidase.

(2) 0.25 ml. 1 % cytochrome *c*; 0.2 ml. cytochrome oxidase.

Manometers 1 and 2 were filled with  $O_2$ - $N_2$  mixtures containing 4 %  $O_2$  and manometer 3 was filled with air. On incubation with formate, and material C, manometer 3 gave an uptake of  $10 \mu l.$  in 30 min. after which the enzyme was inactive. Manometer 1 showed a linear  $O_2$  uptake lasting for  $2\frac{1}{2}$  hr. before the experiment was stopped: the steady  $Q_{O_2} = 29$ . Manometer 2 also showed a small  $O_2$  uptake ( $Q_{O_2} = 13$ ) which decreased slowly after 90 min. None of the controls from which any of the components (enzyme-formate-cytochrome-cytochrome oxidase-oxygen) was missing showed any significant activity. (I am indebted to Dr Hartree of the Molteno Institute, Cambridge, for the concentrated solution of cytochrome *c* and the cytochrome oxidase preparations.)

This reconstruction proves that the formic dehydrogenase of *Bact. coli* reacts with  $O_2$  through the cytochrome system.

**Effect of oxygen on the formic dehydrogenase of *Bact. coli* grown in air.** A culture of *Bact. coli* was grown on the surface of broth agar in air for 20 hr., washed twice and resuspended in distilled water; dry weight of suspension =  $1.2 \text{ mg./ml.}$  Manometers were then set up containing 1 ml. bacterial suspension, 1 ml. phosphate buffer pH 6 and 1 ml.  $M/40$  formate (water in controls); the manometers were filled with  $O_2$ - $N_2$  mixtures containing air, 30, 50, 100 %  $O_2$  respectively. The course of the  $O_2$  uptake at  $37^\circ$  under these conditions is set out in Fig. 10. An oxygen tension greater than that in air has an inactivation effect depending upon its tension. In order to investigate this further, two terms are defined:

**Critical oxygen tension:** the maximum  $O_2$  tension at which the rate of  $O_2$  consumption is not more than halved in 1 hr.

**Protection index:** the number of minutes in which the  $O_2$  consumption is halved when the system is acting in 100 %  $O_2$ .

For the organism grown in air, the critical  $O_2$  tension is that holding in air, 21 %, and the protection index, from Fig. 10, is 25. Both the critical  $O_2$  tension

and the protection index are measures of the degree of protection afforded to the enzyme by the carrier systems present but, since they are determined by

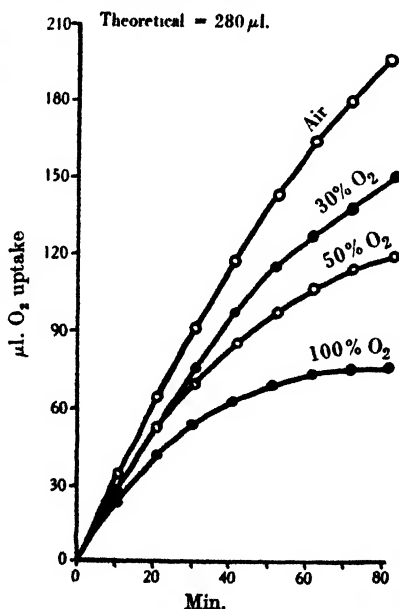


Fig. 10. Oxidation of formate by *Bact. coli* grown in air—effect of  $O_2$  tension on oxygen uptake.



separate experiments, are mutually corroborative. The relevant facts for materials A, B and C are set out in Table VII.

Table VII

Material	$Q_{O_2}$	$Q_{MB}$	Cytochrome content	Critical $O_2$ tension	Protection index
Untreated sediment A	164	110	x x x	15-20 %	20
Autolysed material B	153	324	x	15 %	15
Digested material C	0	474	—	0	0

*Effect of the oxygen tension present during growth of the organism*

In order to grow cultures on the surface of agar under any desired  $O_2$  tension, the following technique was devised: a Roux bottle containing broth-agar is sterilized and, while the agar is still liquid, a small sterile glass boat is slipped in through the neck and embedded in the agar. When cool, the agar is inoculated as usual and 0.5 ml. 10 % NaOH put in the boat. The cotton-wool plug is replaced by a sterile rubber bung fitted with an inlet tube plugged with sterile cotton-wool. The bottle is then connected to a three-way tap connected, on one side to a pump and on the other to an aspirator containing the desired gas mixture. The Roux bottle is then filled with the gas mixture by partial evacuation followed by connexion with the aspirator. If the bottle is one-third evacuated each time and the complete operation repeated some 15 times, almost complete exchange will be effected. When full the bottle is clamped off, disconnected and then reconnected to an aspirator containing  $O_2$ . A water-valve is put in between to prevent mixture of the gases. The whole apparatus is now incubated and as  $O_2$  is used up by the growing organisms, the evolved  $CO_2$  will be absorbed by the NaOH in the boat and  $O_2$  will be sucked in from the aspirator to keep the  $O_2$  tension constant.

In this manner cultures have been grown in the presence of  $O_2$ - $N_2$  mixtures containing 100, 80, 50, 20 and 0 %  $O_2$ . In every case, the cultures have been

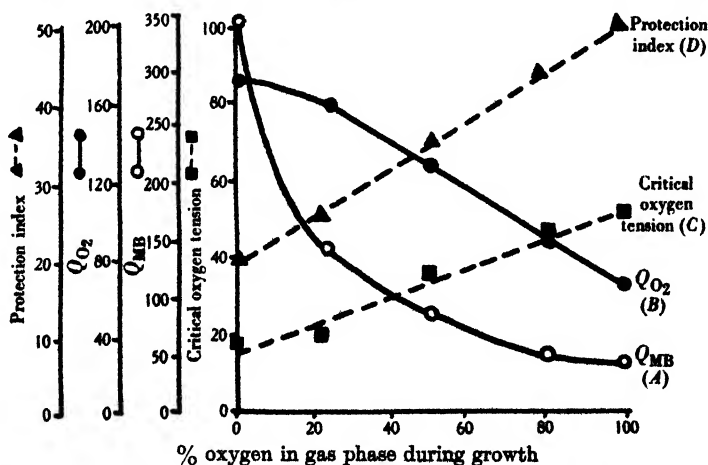


Fig. 11. Effect of oxygen tension present during growth on the properties of *Bact. coli* in regard to formate oxidation. A,  $Q_{MB}$ ; B,  $Q_{O_2}$ ; C, critical oxygen tension; D, protection index.

prepared in washed suspension as usual and the following quantities determined:  $Q_{MB}$ ;  $Q_{O_2}$  in air during linear  $O_2$  consumption over the first 20 min., critical  $O_2$  tension and protection index. The results are plotted in Fig. 11 against the  $O_2$  percentage during growth.

It is seen that increasing the  $O_2$  tension present during growth leads to (1) a decrease in dehydrogenase activity as shown by the drop in  $Q_{MB}$  correlated with (2) a decrease in  $Q_{O_2}$  and (3) an increase in both critical  $O_2$  tension and protection index; Wooldridge *et al.* [1936] have shown that the formic dehydrogenase of *Bact. coli* undergoes negligible variation with the age of the culture so that the results are not complicated by any such effects.

A rise in the critical  $O_2$  tension and the protection index would be brought about, from what has been shown, by a variation in the ratio of carrier systems/enzyme present. Since the cytochrome bands for the organism are weak, it has proved impossible to state whether the cytochrome content of the organism varies significantly according to the manner of growth. However, a culture that has been grown through 20 consecutive anaerobic subcultivations appears to contain essentially the same amount of cytochrome as one grown aerobically for the same period. (I am indebted to Dr T. Mann of the Molteno Institute, Cambridge, for the examination of various cultures with respect to their cytochrome contents.) If, as seems probable, the cytochrome content of the organism is approximately constant then it would follow that any rise in the critical  $O_2$  tolerance would be brought about by inhibition of dehydrogenase activity. From the appearance of curves *A* and *C* (Fig. 11) this would appear to be the case.

When the organism is grown anaerobically, a very high  $Q_{MB}$  is found. This is not due to the presence of formic hydrogenlyase in the anaerobic culture reducing MB through the hydrogenase system as formic hydrogenlyase is not active at the experimental pH 6 used [*v.* Stephenson & Stickland, 1932]. Hence the high  $Q_{MB}$  must be due to a greatly increased production of the dehydrogenase under anaerobic conditions.

*Absence of coenzymes I and II in material C.* Adler & Sreenivasaya [1937] showed that the formic dehydrogenase of certain plants requires the presence of coenzyme I for its action. Table VIII shows the effect of adding coenzymes I and

Table VIII. *Effect of addition of coenzymes on activity of formic dehydrogenase in material C*

Tubes contain: 1 ml. <i>M</i> /10 Na formate. 1 ml. <i>M</i> /20 phosphate buffer pH 6.0. 1 ml. material C=0.45 mg. dry weight. 0.2 ml. 0.5 % methylene blue.	
Additions	Reduction time min. sec.
1 ml. water	9 30
1 ml. cozymase (=0.15 mg. coenzyme I)	9 30
1 ml. coenzyme II prep. (=0.2 mg. coenzyme II)	9 20
1 ml. boiled organism (=20 mg. dry wt.)	12 0

II and boiled organism on the  $Q_{MB}$  of material C in the presence of formate. There is no significant acceleration. Gale & Stephenson [1939] have shown that the malic dehydrogenase of *Bact. coli* requires coenzyme I but that the organism grown on agar possesses only about 27 % of the optimal amount of coenzyme required to activate the dehydrogenase present—hence, in this case, addition of cozymase to the washed suspension produces a considerable acceleration of the rate of reduction of methylene blue. No such effect is obtained with formic dehydrogenase. Further, Yudkin [1933] has shown that the activity of formic dehydrogenase of *Bact. coli* does not vary with the dilution of the organism whereas the activity of other dehydrogenases, known to require coenzymes,

decreases rapidly with dilution. Thus it would appear that the activity of formic dehydrogenase of *Bact. coli* does not depend upon the presence of a coenzyme. Table IX shows the effect of adding 1 ml. of boiled material C containing 20 mg. dry weight to (a) malic dehydrogenase from heart muscle and from *coli*-juice [Gale & Stephenson, 1939] and (b) glucosemonophosphate dehydrogenase from muscle. Also the effects of adding known amounts of coenzymes I and II and boiled *Bact. coli* (20 mg. dry weight) are shown. It is obvious that material C contains no coenzyme I or II. Hence the formic dehydrogenase of *Bact. coli* does not require either coenzyme for its action.

Table IX. *Absence of coenzymes I and II from material C*

(a) Test for coenzyme I

Tubes contain: 1 ml. malic dehydrogenase preparation (a) from heart muscle, (b) from *coli*-juice.  
 1 ml. *M*/10 Na malate.  
 0.2 ml. 2*M* NaCN.  
 1 ml. phosphate buffer pH 7.2.  
 0.2 ml. 0.5 % methylene blue.

Additions	Reduction time	
	(a)	(b)
1 ml. water	> 3 hr.	> 3 hr.
1 ml. cozymase (=0.15 mg. coenzyme I)	1 min. 15 sec.	2 min. 25 sec.
1 ml. boiled material C (=20 mg. dry wt.)	> 3 hr.	> 3 hr.
1 ml. boiled organism (=20 mg. dry wt.)	20 min. 5 sec.	—

(b) Test for coenzyme II

Tubes contain: 1 ml. glucosemonophosphate dehydrogenase preparation.  
 1 ml. phosphate buffer pH 7.5.  
 0.3 ml. *M*/5 glucosemonophosphate.  
 0.2 ml. 0.5 % methylene blue.

Additions	Reduction time
1 ml. water	> 2 hr.
1 ml. coenzyme II (=0.2 mg. coenzyme II)	15 min. 25 sec.
1 ml. boiled material C (=20 mg. dry wt.)	> 2 hr.

### SUMMARY

1. The material obtained by grinding *Bact. coli* in the Booth-Green mill has been investigated for formic dehydrogenase activity.
2. The enzyme resides in the solid part of this material and has not been removed therefrom in an active state.
3. Digestion of this sediment with trypsin gives a material very active with methylene blue in the presence of formate but unable to utilize  $O_2$  as H-acceptor.
4. Spectroscopic examination shows that the formic dehydrogenase of the sediment reacts normally with  $O_2$  through cytochrome *b*. This is confirmed by the effects of cyanide, which inhibits the dehydrogenase in high concentrations.
5. Autolysis of the sediment yields a material which will oxidize formate with oxygen but which is quickly inactivated.
6. Inactivation of the enzyme occurs only when the dehydrogenase is incubated aerobically with formate.
7. The inactivation is not due to the production of oxalic acid, formaldehyde or hydrogen peroxide.
8. The inactivated enzyme can be reactivated by anaerobic incubation. This effect is accelerated by the presence of formate.

9. The inactivation is accelerated by increasing the  $O_2$  tension. If the  $O_2$  tension is decreased sufficiently, the inactivation is abolished.

10. The inactivation is accelerated by the action of cyanide on the carrier system (cytochrome) or checked by the addition of methylene blue.

11. The digested material will react with  $O_2$  in the presence of methylene blue but the extent of the oxidation depends upon (a) the concentration of the dye and (b) the  $O_2$  tension.

12. It is suggested that the enzyme reacts with  $O_2$  along two paths: (1) directly leading to inactivation of the dehydrogenase activity, and (2) indirectly through the carrier system. The latter reaction, by diverting the course of the oxygen, protects the enzyme from the direct oxidation.

13. The digested material will give a steady  $O_2$  uptake with a high concentration of cytochrome *c* and cytochrome oxidase in the presence of a low  $O_2$  tension.

14. *Bact. coli* grown in air can oxidize formate linearly only if the  $O_2$  tension is less than that of air.

15. A method for growing organisms on the surface of agar under any given  $O_2$  tension is described.

16. Increased  $O_2$  tension during growth leads to inhibition of formic dehydrogenase formation.

17. Formic dehydrogenase of *Bact. coli* does not require coenzymes I or II for its action.

The author wishes to thank Prof. Sir F. G. Hopkins for his continued interest in this work and Dr M. Stephenson for her encouragement and help. He is indebted to Dr M. Dixon for valuable criticism and to the Medical Research Council for a personal grant.

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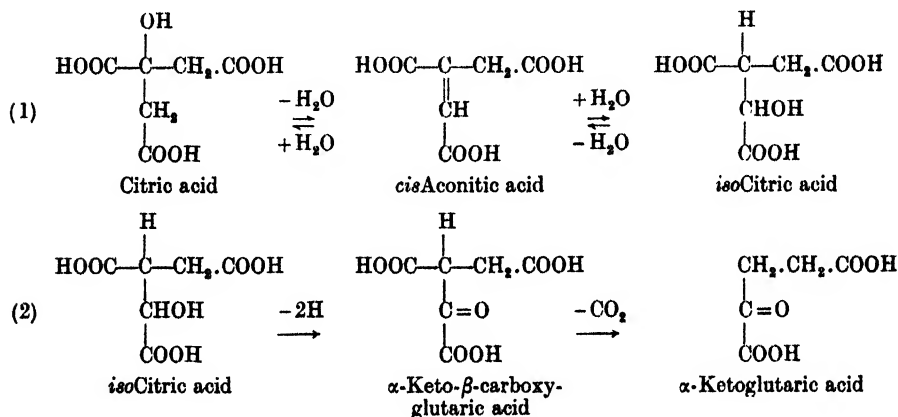
# CXXVI. ISOCITRIC DEHYDROGENASE AND GLUTAMIC ACID SYNTHESIS IN ANIMAL TISSUES

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MARTIUS & KNOOP [1937; Martius, 1937; 1939] have recently shown that the biological breakdown of citric acid proceeds according to the following scheme:



Citric acid first undergoes transformation into *isocitric acid* by the action of the enzyme "aconitase". *isoCitric acid* is then dehydrogenated to the corresponding keto-acid, which is unstable and splits off  $\text{CO}_2$  spontaneously to form  $\alpha$ -ketoglutaric acid. Thus the old term "citric dehydrogenase" designates a mixture of enzymes and the actual dehydrogenation is brought about by an "*isocitric dehydrogenase*".

In connexion with our work on glutamic dehydrogenase and the enzymic synthesis of glutamic acid, i.e. reductive amination of  $\alpha$ -ketoglutaric acid [Euler *et al.* 1938], we were interested in the nature of *isocitric dehydrogenase*, because it catalyses the formation of  $\alpha$ -ketoglutaric acid and thus forms a link between carbohydrate breakdown and protein synthesis in the cells.

The history of "citric dehydrogenase", especially the work of Thunberg, Batelli & Stern, and Bernheim, is given in the monograph of Franke [1934]. More recently Andersson [1933] found that a crude cozymase preparation accelerated the reduction of methylene blue by citric acid and a plant extract, and Wagner-Jauregg & Rauen [1935, 1] described an activation of a similar system by a crude coenzyme prepared according to Warburg & Christian [1935] from red blood cells. Wagner-Jauregg & Rauen [1935, 2] also found that *isocitric acid* was a hydrogen donor for the plant "citric dehydrogenase" system.

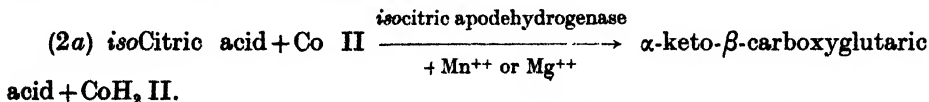
From the point of view of our present knowledge of dehydrogenase systems it was of fundamental interest to find out what coenzyme had been active in

Andersson's and Wagner-Jauregg's experiments. It seems likely that the coenzyme preparations used by these authors contained both codehydrogenase I (cozymase, diphosphopyridinenucleotide, Co I) and codehydrogenase II (Warburg's coenzyme, triphosphopyridinenucleotide, Co II). Using pure preparations of these coenzymes we have found that Co II is the specific coenzyme for isocitric dehydrogenase from animal tissues, higher plants and yeast,<sup>1</sup> while Co I is completely inactive. The bearing of this fact on the problems connected with the importance of the isocitric dehydrogenase in carbohydrate breakdown and amino-acid synthesis is discussed in a later section.

On the basis of the Co II-specificity we were able to study the distribution of the isocitric apodehydrogenase in biological material, to purify the enzyme and to study the dehydrogenating system. The apodehydrogenase was found to be present in all animal tissues so far examined, which makes it probable that citric acid breakdown is part of a general cell reaction. It was possible to obtain the isocitric dehydrogenase free from "aconitase" and thus enzyme preparations were obtained which used isocitric but not citric acid as H donor. It was further shown that in the isolated isocitric dehydrogenase system CO<sub>2</sub> and  $\alpha$ -ketoglutaric acid were formed as the end products, thus confirming Martius and Knoop's scheme.

In experiments with purified apodehydrogenase the system showed a typical "dilution effect", i.e. the rate of the reaction was not proportional to the enzyme concentration, but disappeared more or less completely when the enzyme was diluted to a certain degree. The analysis of this observation led us to the surprising fact that Mn<sup>++</sup> was necessary for the full action of the dehydrogenase; Mg<sup>++</sup> could replace the Mn<sup>++</sup>, but was less active and its optimal concentration was higher than that of Mn<sup>++</sup>. As yet no other dehydrogenase could be found in which Mn or Mg salts had an effect, whilst a number of other enzymic reactions, e.g. transphosphorylation [Ohlmeyer & Ochoa, 1937] and pyruvic acid decarboxylation [Euler *et al.* 1937] by yeast enzymes, are known to be activated by Mn<sup>++</sup>. Concerning the mode of action of these ions in the case of isocitric dehydrogenase it may be possible, that they "link up" the substrate with the apodehydrogenase by salt formation. If the Mn<sup>++</sup> effect involved the reaction between the codehydrogenase and the apodehydrogenase, one would expect that other dehydrogenases would be similarly activated.

According to the results described in this paper reaction (2) of the scheme given above can be written as follows:



Reaction (2b) goes spontaneously and is very fast and therefore the whole reaction (2a + 2b) must go to completion in the direction given by the arrows. That means that in presence of an excess of isocitric acid the total amount of Co II is hydrogenated, and on the other hand, in presence of an excess of Co II, or if the CoH<sub>2</sub> II formed is continuously reoxidized, the total amount of isocitric acid is converted into ketoglutaric acid. Whether reaction (2a) is reversible in analogy with other hydroxy-acid  $\rightleftharpoons$  keto-acid reactions cannot be said, because it was not possible to study this reaction independently of reaction (2b).

<sup>1</sup> The experiments with higher plants and yeast have been done by L. Elliot and will be published separately.

## EXPERIMENTAL

*The components of the system*

(a) *Enzyme preparations.* Acetone-dried heart muscle was used as starting material. Pig heart was freed from fat and ground in a mincer. The pulp was stirred up 3 times with twice the volume of ice-cold acetone, pressed out through muslin each time and finally dried in air by spreading out on filter paper. From the resulting stable preparation enzymes were prepared in different ways.

*Enzyme A.* Acetone-dried tissue was ground in a mortar with sand and 6 times its wt. of water and filtered through muslin on a Büchner funnel. The extract was dialysed for 12 hr. and centrifuged. The enzyme attacks isocitric acid, but not at all or only slowly citric acid.

*Enzyme B.* Fractionation with ammonium sulphate: 70 g. acetone-dried heart muscle were ground twice with 420 ml. 0.1 *M*  $\text{Na}_2\text{HPO}_4$  and sand and pressed out through muslin. The pH of the crude extract (700 ml.) was adjusted to 6.5 by addition of 150 ml. 0.5 *M*  $\text{KH}_2\text{PO}_4$  and 1700 ml. sat. ammonium sulphate solution were added (degree of saturation is 0.66). The protein precipitate was filtered through a thin layer of kieselguhr on a Büchner funnel and redissolved by rubbing up the filter cake with 0.1 *M*  $\text{Na}_2\text{HPO}_4$ . The resulting solution was centrifuged and the supernatant was neutralized with 50 ml. 0.5 *M*  $\text{KH}_2\text{PO}_4$ ; 175 ml. sat. ammonium sulphate were then added and the precipitate formed was discarded. The filtrate was precipitated once more with an equal volume of ammonium sulphate, and the proteins remaining on kieselguhr after filtration were redissolved in 70 ml. water and centrifuged. The reddish, clear solution contains aconitase and a very active isocitric dehydrogenase.

*Enzyme C.* Fractionation with acetone: 50 g. acetone-dried heart muscle were thoroughly ground with 400 ml. water and sand. The extract (250 ml.) was precipitated at 0° with 750 ml. cold acetone; the precipitate was dissolved in 130 ml. water; the centrifuged solution (125 ml.) was precipitated at 0° with 65 ml. acetone and after centrifuging off the precipitate formed, another 65 ml. acetone were added. Both precipitates were dried with cold acetone and ether. The second fraction was used in most of the experiments and is called "enzyme C". The dry powders are nearly completely soluble in water; both fractions are rich in isocitric dehydrogenase but free from aconitase.

(b) *Coenzyme.* Codehydrogenase II was prepared by enzymic phosphorylation of cozymase according to the principle previously described [Euler & Adler, 1938]. The details of this will be published separately.

(c) *Substrate.* isoCitric acid was a synthetic preparation for which we wish to express our thanks to Prof. P. Karrer, Zürich. The Na salt was used in the experiments.

*Citric acid as a substrate*<sup>1</sup>

*Methylene blue as acceptor.* Table I shows that codehydrogenase II and flavinenzyme are necessary for methylene blue reduction when citrate is used as substrate for "enzyme B".

Exps. 2 and 3 show that the methylene blue decoloration with 40  $\mu\text{g}$ . Co II was about 25 times faster than that with 250  $\mu\text{g}$ . of a cozymase preparation of highest purity. It is difficult to say whether cozymase has an action in this system which is 150 times weaker than that of codehydrogenase II or if cozymase is actually inactive and the low rate obtained in Exp. 3 is due to traces of codehydrogenase II present in the cozymase preparation. At any rate the great difference in the degree of action indicates that isocitric dehydrogenase is practically specific for codehydrogenase II.

<sup>1</sup> For a preliminary report see Adler *et al.* [1938, 3].

Table I. The "citric acid dehydrogenase" system

0.5 *M* Na-citrate; "enzyme B"; cozymase (Co I) of highest purity, 1 mg./ml.; codehydrogenase II (Co II), 200  $\mu$ g./ml.; flavinenzyme from yeast, 2.5  $\mu$ g. bound lactoflavin per ml. Each Thunberg tube contained 0.25 ml. 0.5 *M* phosphate buffer, pH 7.6, and in a small inner tube 0.5 ml. 0.02% methylene blue, which is mixed with the other components after evacuation. Temp. 30°.

In Exp. 1 the citrate was added to the other components immediately before the experiment was started; in all other experiments citrate + enzyme + buffer + water were incubated in the open tube for 15 min. at 30°, then the other components were added and the experiment was started.

Exp. no.	Citrate ml.	Enzyme ml.	Co I ml.	Co II ml.	Flavin-enzyme ml.	Decoloration time min.
1	0.25	0.25	—	0.20	0.25	8
2	0.25	0.25	—	0.20	0.25	2
3	0.25	0.25	0.25	—	0.25	53
4	—	0.25	—	0.25	0.25	240
5	—	0.25	0.25	—	0.25	205
6	0.25	0.25	—	0.25	—	79
7	0.25	0.25*	—	0.25	0.25	240
8	0.25	0.25	—	0.25	0.25†	78
9	0.25	0.25	—	0.25‡	0.25	240

\* Boiled after incubation with citrate.

† Boiled flavinenzyme.

‡ Alkali-heated Co II.

A comparison between Exps. 1 and 2 shows that the decoloration time is remarkably shorter when the citrate is incubated with the enzyme for some time before the other components are added and the dehydrogenation is started. This effect is explained by the Martius-Knoop scheme: during incubation the transformation citric acid  $\rightleftharpoons$  isocitric acid takes place and possibly the equilibrium is reached within the incubation time, whilst in the experiment without incubation there is not enough isocitric acid formed to reach the optimal concentration for the dehydrogenase reaction.

*Oxygen as acceptor.* Fig. 1 shows that the system citrate + enzyme B + codehydrogenase II takes up  $O_2$  when it is completed by flavinenzyme and methylene blue.

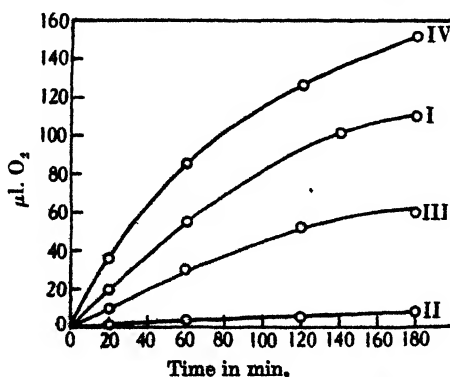


Fig. 1. Aerobic breakdown of citric acid. Curve I: 0.25 ml. *M*/2 citrate, 1 ml. "enzyme B", 0.5 ml. flavinenzyme, 0.25 ml. veronal buffer pH 7.66, 0.5 ml. methylene blue 1:5000; after 15 min. incubation at 30°, 0.25 ml. (=25  $\mu$ g.) Co II were added from the side bulb of the manometer flask. Curve II: with heated flavinenzyme. Curve III: without methylene blue. Curve IV: with double amount of flavinenzyme. No  $O_2$  uptake was obtained in controls without substrate, without Co II, with heated enzyme and with Co I instead of Co II. The centre cup of the vessels contained 0.2 ml. 10% KOH.



In the absence of methylene blue the  $O_2$  uptake is slower, because then the rate is limited by the autoxidizable fraction of our flavinenzyme preparation, i.e. the flavinphosphate protein fraction, whilst the flavin-adenine-dinucleotide protein fraction [Warburg & Christian, 1938; Haas, 1938] will react rapidly only if methylene blue is added. In absence of flavinenzyme the reaction is extremely slow; this means that "enzyme B" does not contain appreciable amounts of "diaphorase II" [Adler *et al.* 1939], a flavoprotein of animal tissues, which transports hydrogen from  $CoH_2$  II to acceptors like methylene blue. This conclusion was confirmed by direct spectrophotometric determination in the system  $CoH_2$  II + "enzyme B" +  $O_2$ ; with the same technique it was shown that "enzyme B" was relatively rich in "diaphorase I", the  $CoH_2$  I-specific hydrogen transporting enzyme.

#### *Separation of aconitase and isocitric dehydrogenase*

When "enzyme B" was dialysed in a cellophane tube against running water for 20 hr., the activity towards citric acid was practically abolished; the activity towards isocitric acid was decreased too, but could be restored to the level of the non-dialysed enzyme by addition of  $Mn^{++}$  (cf. p. 1037), whilst citrate was not attacked even in presence of  $Mn^{++}$  or of boiled undialysed enzyme; but when citrate was incubated with non-dialysed "enzyme B" for 15 min., the mixture deproteinized by heating and used as a substrate in a Thunberg experiment containing the dialysed "enzyme B", the MB was rapidly decolorized. Therefore, the aconitase must have been inactivated by dialysis.

Another way of separating aconitase is to precipitate the crude enzyme with acetone. Thus "enzyme C" which is a stable acetone powder, is completely inactive with citrate though highly active with isocitrate, especially after addition of  $Mn^{++}$ . Sensitivity to acetone treatment is also characteristic of fumarase [Clutterbuck, 1928], but according to Martius [1939] aconitase and fumarase are different enzymes.

#### *The isocitric dehydrogenase system*

##### *(a) Methylene blue as acceptor.*

The dehydrogenation of isocitric acid by one of the enzymes A, B, or C with methylene blue as H acceptor needs the addition of the same components as if citric acid is used as a substrate, namely codehydrogenase II and flavinenzyme. Codehydrogenase II cannot be replaced by cozymase.

Fig. 2 shows the dependence of the rate of methylene blue reduction on the codehydrogenase II concentration and in Fig. 3 the influence of increasing amounts of yeast flavinenzyme is shown. The relation between the rate of isocitric acid dehydrogenation and the amount of apodehydrogenase is described in a later section in connexion with the experiments on  $Mn^{++}$  activation. Here it may be mentioned that proportionality between rate and apodehydrogenase concentration exists only if the system contains an optimal amount of  $Mn^{++}$ .

*Substrate affinity.* Experiments on the influence of isocitric acid concentration showed that the rate was still optimal when the concentration of the substrate was as low as  $5 \times 10^{-5} M$ , assuming that the isocitric acid preparation used contained 50% of the natural form. Thus, in a Thunberg experiment with 2.0 ml. total volume, 19  $\mu g.$  isocitric acid (equivalent to 37  $\mu g.$  methylene blue) were sufficient to decolorize the methylene blue (20  $\mu g.$ ) at the optimal rate. Since a further decrease in the amount of methylene blue would have introduced considerable error in the measurement of the decoloration time, experiments

with still lower amounts of *isocitric* acid could not be done. In spectrophotometric experiments (cf. p. 1036), the *isocitric* acid concentration could be lowered to  $1.25 \times 10^{-5} M$ , without a distinct decrease in the rate of dehydrogenation. Thus, the Michaelis constant  $K_m$  must have a value  $< 1.25 \times 10^{-5} M$ , which means that the affinity of *isocitric* acid for the apodehydrogenase is extremely high.

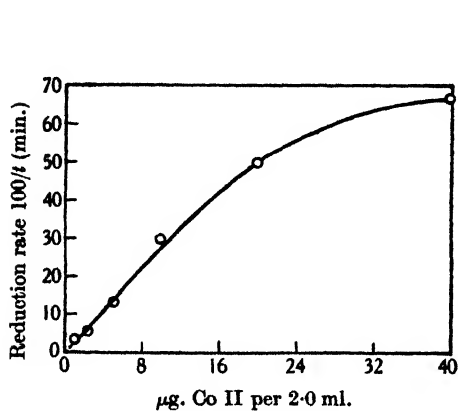


Fig. 2.

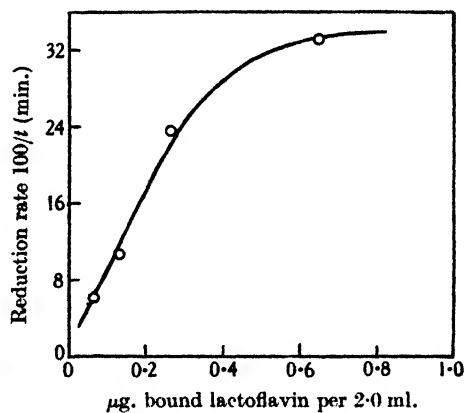


Fig. 3.

Fig. 2. *isoCitric* acid dehydrogenation; effect of coenzyme concentration. Thunberg technique. 0.1 ml. "enzyme B" was used in each experiment.

Fig. 3. *isoCitric* acid dehydrogenation; effect of flavinenzyme concentration. Thunberg technique. 0.1 ml. "enzyme B" was used in each exp.

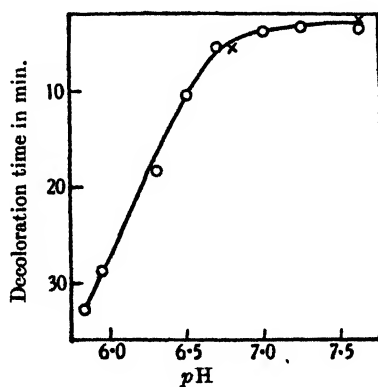


Fig. 4. *isoCitric* acid dehydrogenation; effect of pH. Thunberg technique. 0.1 ml. "enzyme C", 20 μg. Co II, 0.25 ml. flavinenzyme and 2 mg. *isocitric* acid were used in each exp. O = veronal buffer; x = glycine buffer.

**Influence of pH.** Fig. 4 shows the influence of the pH of the solution on the rate of *isocitric* acid dehydrogenation, determined by the Thunberg technique. It is important to say that phosphate buffer could not be used in these experiments as it was found that phosphate ions inhibit the dehydrogenase reaction (cf. p. 1040) and that the % inhibition varies considerably with the pH. Therefore, the pH curve with phosphate buffer showed a maximum between pH 6 and 6.5, where the phosphate inhibition is relatively small, and a minimum between pH 7 and 8, where phosphate exerts a strong inhibiting action. By the use of

veronal and glycine buffer this difficulty was overcome and a normal pH curve, analogous to other animal dehydrogenases was found. The rate is high at pH 7-7.5 and falls off rapidly below pH 6.5; the values at alkaline reaction, where the curve also falls off, are not shown in the figure, because in this region the rate may be decreased by destruction of the codehydrogenase.

(b) *Oxygen as acceptor.*

*The components of the system.* A mixture of isocitric acid, apodehydrogenase and codehydrogenase II takes up  $O_2$  if the transfer of H from the reduced coenzyme to the  $O_2$  is made possible by the addition of flavinenzyme. A further addition of methylene blue increases the rate of  $O_2$  uptake very much, because in presence of this dye even the "new", i.e. not readily autoxidizable, flavoprotein, present in the flavinenzyme preparation, is utilized (Table II).

Table II. *Aerobic dehydrogenation of isocitric acid*

The total system contained 0.25 ml. apodehydrogenase solution (corresponding to 4 mg. acetone powder "enzyme C"); 0.20 ml. (=20  $\mu$ g.) codehydrogenase II; 0.25 ml. flavinenzyme from yeast (corresponding to 0.8  $\mu$ g. bound lactoflavin); 0.50 ml. methylene blue 1:1000; 0.25 ml. veronal-acetate buffer (Michaelis), pH 7.66, and 0.25 ml. Na isocitrate (corresponding to 5 mg. isocitric acid); the total volume was 2.25 ml. The centre cup of the Warburg vessels contained 0.3 ml. 7% KOH, absorbed on filter paper. The substrate was added from the side bulb.

	$\mu$ l. $O_2$ in 20 min.
Total system	49
No substrate	0
With heated enzyme	0
No coenzyme	0
No flavinenzyme	3
No methylene blue	8
With Co I instead of Co II	0

*The oxygen equivalence.* The amount of  $O_2$  taken up by a certain amount of our isocitric acid preparation agrees rather well with the theory: one pair of H atoms is taken away from the substrate, dihydrocoenzyme is formed and the 2H transported to the  $O_2$ , giving  $H_2O_2$ ; the latter is split by catalase, the presence of which in the apodehydrogenase preparation was shown in separate experiments, and 1/2 mol.  $O_2$  is liberated again. Thus an actual uptake of 1/2 mol.  $O_2$  per mol. isocitric acid would be expected.

We found in several experiments with "enzyme C" an  $O_2$  uptake a little higher than calculated, e.g. for 1.9 mg. isocitric acid an  $O_2$  uptake of 65  $\mu$ l. (calculated 56  $\mu$ l.) and for 3.8 mg. isocitric acid an oxygen uptake of 125  $\mu$ l. (calculated 112  $\mu$ l.) was found. The small discrepancy between the theoretical and the experimental values can have several reasons; it can be at least partially explained by the fact that our isocitric acid preparation contained an unknown amount of the corresponding lactone which has a lower mol. wt. and so will give higher values for the  $O_2$  uptake than the same amount of free acid. With crude enzyme preparations, e.g. "enzyme A", the  $O_2$  uptake was usually lower than calculated, which possibly indicates that part of the reduced coenzyme is used up in an anaerobic reaction; one could assume that cis-aconitic acid which, in presence of crude enzymes, is in equilibrium with isocitric acid, might act as H acceptor, forming tricarballic acid. However, no direct proof of the existence of such a reaction has been found as yet.

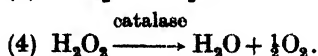
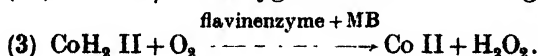
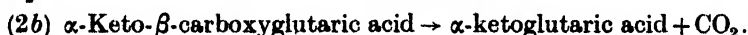
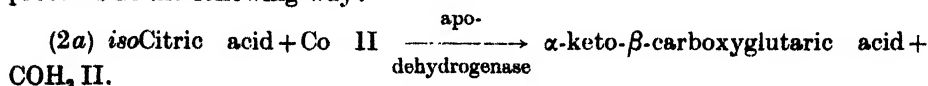
*$CO_2$  as a reaction product.* If in the aerobic experiment no KOH is used in the centre cup, the negative pressure is replaced by a positive one, showing that  $CO_2$

is formed during the reaction. The respiratory quotient was determined according to Warburg & Yabusoe [1924]. In experiments in which the reaction mixture was the same as that given in Table I, but with 4 mg. substrate, after 100 min., when the reaction was finished, 140  $\mu$ l.  $O_2$  had been taken up and 278  $\mu$ l.  $CO_2$  had been formed. Thus, the quotient mol.  $CO_2$ /mol.  $O_2$  = 2, i.e. for each mol. isocitric acid 1/2 mol.  $O_2$  is taken up and 1 mol.  $CO_2$  is formed. This result is in complete agreement with Martius and Knoop's scheme.

*$\alpha$ -Ketoglutaric acid as a reaction product.* Definite proof of the mechanism of the isolated dehydrogenase reaction as given by scheme (2) consists in the isolation of  $\alpha$ -ketoglutaric acid.

The collected reaction mixtures of five aerobic experiments, corresponding to those given in Table II, were deproteinized with 2% trichloroacetic acid and centrifuged. The supernatant solution was freed from methylene blue by slow filtration through a layer of kieselguhr and concentrated *in vacuo* to 5 ml. After addition of 3 ml. sat. 2:4-dinitrophenylhydrazine in 2N HCl, crystals of a 2:4-dinitrophenylhydrazone settled out. They were collected after standing overnight and washed with 2N HCl. For purification the hydrazone was taken up in diluted  $Na_2CO_3$ , in which it was completely soluble, and reprecipitated with HCl. The substance was identified as the 2:4-dinitrophenylhydrazone of  $\alpha$ -ketoglutaric acid by m.p. (218°) and mixed m.p. (218°); the 2:4-dinitrophenylhydrazone of the pure acid melted at 219°. The yield was 12 mg. of the crude substance.

Summarizing the preceding qualitative and quantitative results, it has been shown that the aerobic dehydrogenation of isocitric acid by the isolated system proceeds in the following way:



The sum of these reactions will be



The catalytic components of the system which brings about the primary anaerobic step of the dehydrogenation, i.e. isocitric apodehydrogenase and cohydrogenase II, are present probably in all animal cells. But for the transport of H from  $\text{CoH}_2 \text{ II}$  to  $O_2$  we used flavinenzyme from yeast, because our apodehydrogenase preparation did not contain a carrier enzyme capable of reacting with  $\text{CoH}_2 \text{ II}$ . However, as Adler *et al.* [1939] have shown, animal tissues do in fact contain an enzyme, "diaphorase II", which transfers H from  $\text{CoH}_2 \text{ II}$  to acceptors like methylene blue and probably also to cytochrome; hence the possibility of aerobic dehydrogenation of isocitric acid in animal tissues is evident. Actually, citric acid has been shown to increase the  $O_2$  uptake of various animal tissues [Batelli & Stern, 1911; Krebs & Eggleston, 1938].

Besides the aerobic way there is another possibility for continuous dehydrogenation of isocitric acid in the cells, namely the anaerobic reoxidation of the  $\text{CoH}_2 \text{ II}$  by iminoglutaric acid, i.e. by  $\alpha$ -ketoglutaric acid +  $\text{NH}_3$ , catalysed by glutamic apodehydrogenase. This reaction will be discussed in a later section.

(c) *Spectrophotometric experiments.*

The primary reaction (2a) between *isocitric* acid and codehydrogenase II in presence of the apodehydrogenase can be easily studied by spectrophotometric determination of the characteristic absorption band with maximum at 340 m $\mu$  of the dihydro-codehydrogenase II.

In Fig. 5, Curve I shows the hydrogenation of a certain amount of codehydrogenase II by an excess of *isocitric* acid, catalysed by "enzyme C". In the parallel experiment (Curve II), the same amount of Co II was used, but the hydrogenation was brought about by hexosemonophosphate in presence of hexosemonophosphate apodehydrogenase from yeast. This reaction is known to be irreversible, i.e. if an excess of substrate is used, the total amount of coenzyme is hydrogenated. The end extinctions in Exps. I and II are equal; thus the hydrogenation of the Co II was complete in the *isocitric* system.

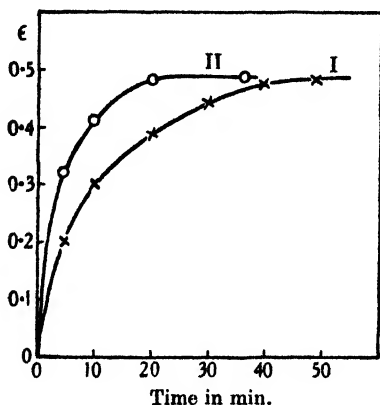


Fig. 5.

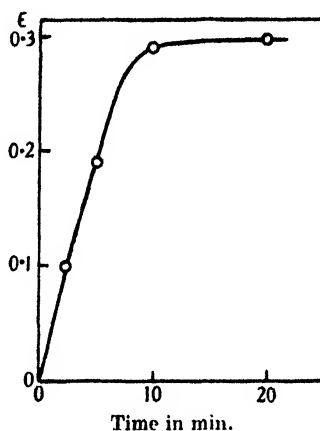


Fig. 6.

Fig. 5. Hydrogenation of codehydrogenase II by excess of *isocitric* acid (Curve I) and hexosemonophosphate (Curve II). Curve I: 0.19 mg. ( $\approx 10^{-3}$  mM) *isocitric* acid, 0.1 ml. "enzyme C", 0.5 ml. ( $\approx 0.17 \times 10^{-3}$  mM) Co II, 0.5 ml. veronal buffer. Curve II: 0.2 ml. *M/10* hexosemonophosphate, 0.1 ml. hexosemonophosphate dehydrogenase, 0.5 ml. Co II, 0.3 ml. *M/2* phosphate buffer. Total volume 4 ml. The extinction ( $\epsilon$ ) at  $\lambda = 334$  m $\mu$ , indicating the formation of  $\text{CoH}_2$  II, was measured photoelectrically.

Fig. 6. Hydrogenation of Co II by less than the equivalent amount of *isocitric* acid.  $0.2 \times 10^{-3}$  mM *dl-isocitric* acid and  $0.13 \times 10^{-3}$  mM. Co II were used.  $\text{CoH}_2$  II found:  $0.11 \times 10^{-3}$  mM (calc.  $0.10 \times 10^{-3}$ ).

When an excess of codehydrogenase was used, the dihydro-compound formed was equivalent to the *isocitric* acid (Fig. 6).

These experiments show that the reaction between *isocitric* acid and Co II goes to completion. This can be explained by the following alternative assumptions: (1) the primary reaction (2a) is irreversible; (2) reaction (2a) is reversible and gives an equilibrium, which is rapidly disturbed by the decomposition of the keto-acid formed (2a + 2b). The observation that in aerobic experiments  $\text{CO}_2$  output begins immediately shows that the  $\alpha$ -keto- $\beta$ -carboxyglutaric acid actually is rapidly decarboxylated. Thus, the conditions for a reaction according to the second assumption seem to be given. Then, concerning the rate of the spontaneous decarboxylation, it could be said that it must be rather high, because in spectrophotometric as well as in methylene blue experiments the rate

of dehydrogenation seemed to be dependent solely on the enzyme concentration, even when this was relatively high. If the decarboxylation were a slow reaction, and if assumption (2) were correct, it should have limited the rate of the dehydrogenation. A more detailed study of the kinetics of this reaction would be of interest.

Fig. 7 represents a spectrophotometric proof of the Co II-specificity of isocitric apodehydrogenase. Pure cozymase (Co I) gave no reduction band, but after addition of Co II the extinction was raised to a value corresponding to complete transformation into  $\text{CoH}_2\text{II}$ . When pyruvate was added at the end of the reaction, no change occurred. This rules out the possibility that the reaction might involve a dephosphorylation of  $\text{CoH}_2\text{II}$  to  $\text{CoH}_2\text{I}$ , because the latter compound would have been reoxidized by the pyruvate and lactic apodehydrogenase which was present in the enzyme preparation.

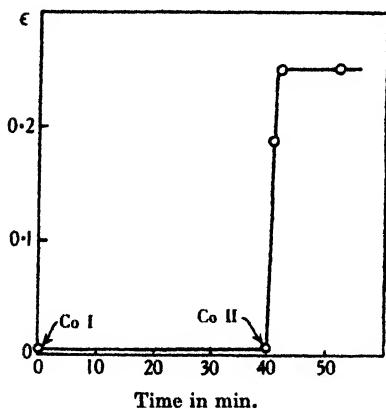


Fig. 7.

Fig. 7. Codehydrogenase II-specificity of isocitric dehydrogenase.

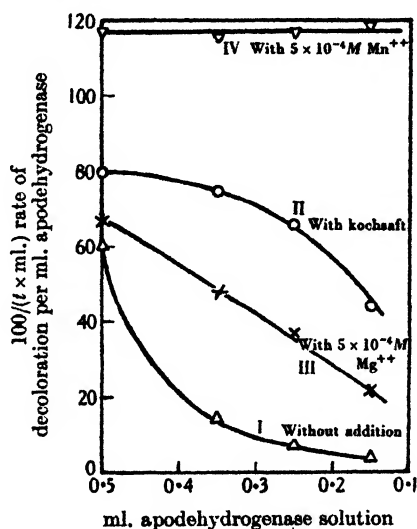


Fig. 8.

Fig. 8. Effect of enzyme-dilution; activation by kochsaft,  $\text{Mg}^{++}$  and  $\text{Mn}^{++}$ . The curves refer to the Thunberg exp. of Table III.

#### *$\text{Mn}^{++}$ and $\text{Mg}^{++}$ as complements of the isocitric dehydrogenase*

**Methylene blue experiments.** As mentioned before, it was observed that the isocitric dehydrogenase system showed a typical "dilution effect", especially if purified enzymes were used. This may be illustrated by the experiments given in Table III and by Curve I in Fig. 8. The table contains the original figures for the decoloration times obtained with different amounts of apodehydrogenase, whilst in the curves of Fig. 8 the reciprocal values of the decoloration times, calculated for 1 ml. apodehydrogenase solution, are plotted against the amounts of apodehydrogenase solution used. It is seen that Curve I falls off sharply with decreasing amounts of apodehydrogenase; if there had been no dilution effect the curve would have remained horizontal.

From this observation one must conclude that the apodehydrogenase solution contained besides the enzyme another substance, essential for the reaction and

present in a suboptimal concentration. If this substance were thermostable, then kochsaft of the apodehydrogenase solution, added to the system, should be able to remove the dilution effect. In fact, kochsaft caused a great activation, especially at low enzyme concentrations, i.e. it partially removed the dilution effect. Further, it was shown that the ash of the apodehydrogenase preparation still activated the reaction; the activating substance must therefore have been inorganic. Mn salt was then found to activate enormously and to bring about a complete proportionality between rate and apodehydrogenase concentration. This is demonstrated in Table III and by Curve IV in Fig. 8.  $Mg^{++}$  had a similar action but the activation was less and a dilution effect was still found.

Table III. *Effect of  $Mn^{++}$  and  $Mg^{++}$  on the isocitric dehydrogenase*

The apodehydrogenase solution used contained 1.8 mg. "enzyme C" per ml. The reaction mixture contained 0.1 ml. (=0.19 mg.) isocitric acid as Na salt, apodehydrogenase solution in the varying amounts given in the first column, 0.20 ml. (=0.20  $\mu g$ .) Co II, 0.25 ml. flavinenzyme, 0.25 ml. veronal buffer, pH 7.66, 0.5 ml. methylene blue 1:5000, plus various additions as indicated. Total volume 2.0 ml.

Apodehydro- genase ml.	Decoloration time				
	No addition min. sec.	Kochsaft from 3.6 mg. "enzyme C" min. sec.	Ash from 1.5 mg. "enzyme C" min.	$MgSO_4$ $5 \times 10^{-4} M$ min. sec.	$MnSO_4$ $5 \times 10^{-4} M$ min. sec.
0.50	3 20	2 30	—	3 0	1 45
0.35	20 0	3 50	—	6 0	2 30
0.25	52 0	6 0	11	10 50	3 20
0.15	140 0	16 0	—	30 30	5 30

Controls with  $Mg^{++}$  and  $Mn^{++}$  and without substrate or without Co II or without flavinenzyme were negative.

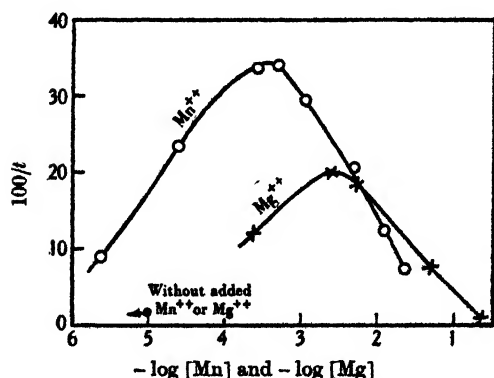


Fig. 9.

Fig. 9. Effect of  $Mg^{++}$  and  $Mn^{++}$  concentration. Thunberg experiments with 0.25 ml. "enzyme C" (=0.45 mg. dry powder) and the other components as given in Table III.

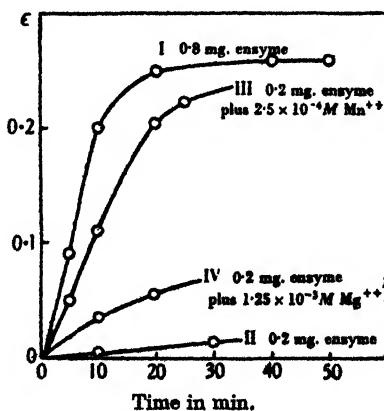


Fig. 10.

Fig. 10. Effect of enzyme dilution and of  $Mn^{++}$  and  $Mg^{++}$  on the rate of  $CoH_2$  formation. 0.2 mg. isocitric acid and 70  $\mu g$ . Co II were used in all experiments. Amounts of "enzyme C" and additions of  $MnSO_4$  and  $MgSO_4$  as indicated.

Fig. 9 shows that the activating action of Mn and Mg salts is dependent on their concentration. For both ions distinct concentration optima exist, which

were found to be about  $5 \times 10^{-4} M$  for  $Mn^{++}$  and about  $2.5 \times 10^{-3} M$  for  $Mg^{++}$ . The maximal activity brought about by  $Mg^{++}$  is only 59 % of that caused by  $Mn^{++}$ .

If suboptimal amounts of  $Mn^{++}$  and  $Mg^{++}$  are added simultaneously, the activity was only very slightly higher than the activity found with  $Mn^{++}$  alone. This seems to indicate that  $Mn^{++}$  and  $Mg^{++}$  are involved in the same reaction and their action is in principle the same, but  $Mn^{++}$  may have a greater affinity for the enzyme.  $CaCl_2$ ,  $ZnSO_4$  and  $CdSO_4$  had no effect.

As is known, Mg and Mn ions are normal constituents of tissues, and therefore it is likely that these ions actually represent the natural complements of the isocitric dehydrogenase system.

*Spectrophotometric experiments.* The spectrophotometric determination of the rate of  $CoH_2$  II-formation shows that the  $Mn^{++}$  and  $Mg^{++}$  effect is involved in the primary step of the dehydrogenation and has nothing to do with the transport of H from  $CoH_2$  II to the acceptor. Curve I in Fig. 10 gives the rate of  $CoH_2$  II-formation with 0.8 mg. of the acetone powder "enzyme C". When 1/4 of this amount was used (Curve II), the rate was much less than 1/4 of the rate given in Curve I: within 10 min. only about 1/15 as much  $CoH_2$  II was formed. Thus, the "dilution effect", shown with the Thunberg technique, is confirmed by the spectrophotometric experiment. Curve III shows the effect of  $Mn^{++}$  and Curve IV that of  $Mg^{++}$  on the rate of  $CoH_2$  II-formation with the lower apodehydrogenase concentration.

Concerning the mechanism of  $Mn^{++}$  and  $Mg^{++}$  action it has been already mentioned that the most probable assumption may be that the ions favour the combination of apodehydrogenase and substrate. We have tested a number of other dehydrogenase systems, including lactic, malic, glutamic and hexosemonophosphate dehydrogenases, without finding any Mn or Mg effect. Therefore, the effect probably does not involve the equilibrium apodehydrogenase + codehydrogenase  $\rightleftharpoons$  holodehydrogenase, which occurs in all these systems. Another possibility to be taken into consideration is an acceleration of the decarboxylation of the primary reaction product,  $\alpha$ -keto- $\beta$ -carboxyglutaric acid, by  $Mn^{++}$  and  $Mg^{++}$ . According to the assumption discussed on p. 1037, the primary dehydrogenation reaction (2a) is reversible and proceeds to completion only if the keto-acid decomposes. Therefore, dependence of the decarboxylation on  $Mn^{++}$  would result in the formation of only a small amount of  $CoH_2$  II in systems deprived of  $Mn^{++}$ . There is, however, experimental evidence against the view that  $Mn^{++}$  affects the decarboxylation. If that were the case, the dehydrogenation should proceed, even in the absence of  $Mn^{++}$ , if a ketone fixative is added to combine with the keto-acid and so disturb the assumed equilibrium (2a). With the spectrophotometric technique it was, however, shown that dimedon does not accelerate the  $CoH_2$  II-formation in a  $Mn^{++}$ -poor system, but that addition of  $Mn^{++}$  then had the known effect.

### Inhibitors

*Iodoacetic acid.* The rate of methylene blue decoloration in the complete isocitric dehydrogenase system was inhibited 97 % by  $M/100$  and 75 % by  $M/1000$  iodoacetic acid. This effect was confirmed with the spectrophotometric technique, although here higher concentrations of iodoacetic acid seemed to be necessary to bring about a similar inhibition.

Thus isocitric dehydrogenase belongs to the group of apodehydrogenases which are inhibited by iodoacetic acid: triosephosphate and alcohol apodehydro-



genase from yeast [Adler *et al.* 1938, 2], triose dehydrogenase of animal tissues [Green *et al.* 1937], succinic dehydrogenase [Hopkins *et al.* 1938] and, to a less extent, alcohol dehydrogenase from liver [Adler *et al.* 1938, 2] and lactic dehydrogenase from yeast (unpublished experiment). All the other dehydrogenases were found to be resistant to iodoacetic acid. According to our present knowledge, sensitivity to this substance indicates the presence in the enzyme molecule of SH-groups which are essential for activity.

*Pyrophosphate.* The isocitric acid dehydrogenation is strongly inhibited by pyrophosphate; addition of  $Mn^{++}$  can, however, abolish this inhibition (Table IV).

Table IV. *Pyrophosphate inhibition*

Thunberg experiments with 0.2 mg. isocitric acid, 4 mg. "enzyme C", 20  $\mu$ g. Co II, flavin-enzyme, veronal buffer. Total volume 2.0 ml.

Addition	Decoloration time	
	min.	sec.
	2	30
0.2 ml. $M/10 Na_4P_2O_7$ (neutralized)	20	0
$5 \times 10^{-4} M Mn^{++}$	2	0
$Na_4P_2O_7$ plus $Mn^{++}$	2	0

From these experiments it becomes clear that the pyrophosphate inhibition is due to combination with the  $Mn^{++}$  or  $Mg^{++}$  present in the enzyme preparation.

As shown by Leloir & Dixon [1937], pyrophosphate also inhibits succinic dehydrogenase and it is suggested by these authors that pyrophosphate may compete with the substrate for the affinity centres of the enzyme, just as is assumed for malonate. Our experience with isocitric dehydrogenase indicated that the pyrophosphate inhibition of succinic dehydrogenase might be due to Mn or Mg removal. However, in connexion with other experiments, it was found in this institute [Euler & Hellström, 1939], that Mn did not influence the succinic dehydrogenase. Thus, the mechanism of pyrophosphate inhibition actually seems to be different in the two systems.

*Phosphate.* As mentioned before (cf. p. 1033), phosphate exerts a distinct inhibition, which is higher in the alkaline than in the acid region (Table V).

Table V. *Phosphate inhibition*

0.1 ml. "enzyme B" and different amounts of phosphate buffer were used; the other components were the same as given in Table IV. The pH was determined after the experiment.

pH	Phosphate concentration ( $M$ )	Rate of MB- decoloration (100/t)	Inhibition %
7.56	$1.25 \times 10^{-3}$	16	—
7.54	$1.88 \times 10^{-3}$	10	37.5
7.58	$2.50 \times 10^{-3}$	5	56
7.56	$6.25 \times 10^{-3}$	2.8	82
6.64	$1.25 \times 10^{-3}$	11.8	—
6.63	$2.50 \times 10^{-3}$	8.7	26
6.63	$6.25 \times 10^{-3}$	7.7	34

Phosphate inhibition was observed by Theorell [1935] in the complete hexosemonophosphate system and confirmed by Adler & Günther [1938] by the spectrophotometric method, thus showing that phosphate affects the primary reaction between substrate, apo- and co-dehydrogenase. A competition by the phosphate with either the substrate or the Co II for the apoenzyme may be the

reason for the inhibition. Similarly, in the *isocitric* system, phosphate may compete with the Co II. Furthermore, as manganese phosphate is only slightly soluble, the phosphate inhibition could be explained by precipitation of  $Mn^{++}$ .

No inhibition was found with cyanide, malonate, fluoride and oxalate.

### Distribution

*isoCitric* apodehydrogenase has been found in all animal tissues so far examined. The tissues were minced and dried with acetone, the acetone preparations were extracted by grinding with 5 times the weight of water and sand and the extracts were dialysed for 5–20 hr. In the cases of brain and spleen the apodehydrogenase was not found in extracts prepared in this way, but its presence could be shown in extracts from fresh tissue. Table VI gives the relative amounts of the apodehydrogenase in various tissues as determined by Thunberg experiments. Mn salt was added to the reaction mixture.

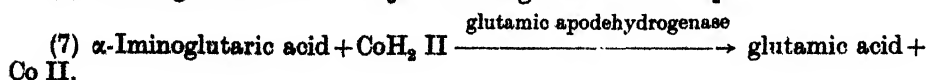
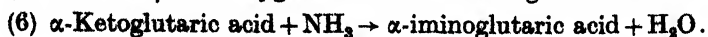
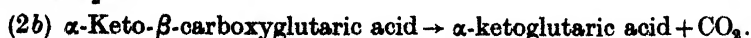
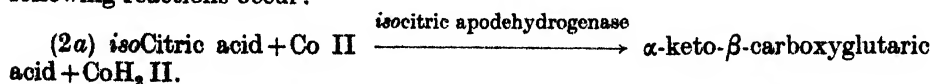
Table VI. *Distribution of isocitric apodehydrogenase*

Tissue	Relative concentration
Heart	+++
Liver	+++
Kidney	+++
Adrenal gland	+++
Ovary	++
Intestine	++
Muscle	+
Brain	+
Lung	+
Testis	+
Jensen sarcoma [cf. Euler <i>et al.</i> 1939]	+
Spleen	(+)

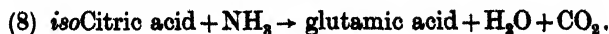
The ratio of the activities of *isocitric* and glutamic apodehydrogenases is different for different tissues. Furthermore, the activities of two purified enzyme solutions, the one prepared from liver and the other from heart muscle, were determined spectrophotometrically in the systems (a) *isocitric* acid + Co II, and (b)  $CoH_2 II$  + ketoglutaric acid +  $NH_3$ . When the two enzymes were diluted in such a way that they had the same activity in system (a), the ratio of their activities in system (b) was 1:4. Therefore *isocitric* and glutamic apodehydrogenases are not identical.

### The formation of glutamic acid from isocitric acid

Recently it was shown that the specific glutamic dehydrogenase catalyses the reductive amination of  $\alpha$ -ketoglutaric acid [Euler *et al.* 1938]. Both Co I and Co II were found to act as coenzymes of this apodehydrogenase. Therefore, it follows that *isocitric* acid can be converted to glutamic acid if *isocitric* apodehydrogenase, Co II, glutamic apodehydrogenase and  $NH_3$  are present. The following reactions occur:



The sum of these reactions is



Thus, in the *isocitric* system, not only the substrate for the glutamic acid synthesis but also the H in the form of  $\text{CoH}_2$  II is available for the reductive amination. The reaction represents an oxidoreduction between *isocitric* acid and  $\alpha$ -iminoglutaric acid, catalysed by Co II, which oscillates between the two specific apodehydrogenases. This is a new example of the validity of the "two enzyme scheme" for coenzyme-dependent oxidoreductions, which was proposed previously for the oxidoreductions between triosephosphate and acetaldehyde or pyruvic acid and similar reactions [cf. Euler *et al.* 1936; Adler *et al.* 1938, 1].

The experimental evidence for the mechanism given above is based on the observation that not only *isocitric* but also glutamic dehydrogenase uses Co II as coenzyme. It has been shown [Euler *et al.* 1938] that glutamic apodehydrogenase from liver catalyses the dehydrogenation of both  $\text{CoH}_2$  I and  $\text{CoH}_2$  II by iminoglutaric acid. Since then we have been able to confirm this fact with glutamic apodehydrogenase from other animal tissues. Thus, it becomes clear that the non-specificity of glutamic apodehydrogenase with respect to the codehydrogenases is common for animal tissues and that reaction (8) can be a general cell reaction for the animal body. These facts are demonstrated by the following spectrophotometric experiments.

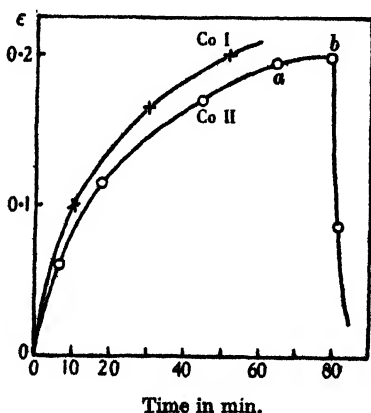


Fig. 11.

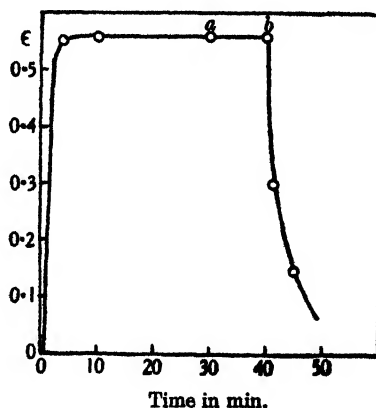


Fig. 12.

Fig. 11. Codehydrogenases I and II as coenzymes of glutamic dehydrogenase. 0.5 ml.  $M/2$  glutamate, 0.1 ml. dialysed aqueous extract from acetone-liver, 0.2 ml. Co I or Co II. In the experiment with Co II were added: at (a) 0.1 ml.  $M/2$  acetaldehyde, at (b) 0.1 ml.  $M/10$  ketoglutarate plus 0.1 ml.  $M/2$   $\text{NH}_4\text{Cl}$ .

Fig. 12. Hydrogenation of Co II by *isocitric* acid and dehydrogenation of the  $\text{CoH}_2$  II by  $\alpha$ -iminoglutaric acid. 0.1 ml.  $M/10$  *isocitrate*, 0.1 ml. dialysed aqueous extract of acetone kidney, 145  $\mu\text{g}$ . Co II. At (a) 0.1 ml.  $M/10$  ketoglutarate, at (b) 0.1 ml.  $M/2$   $\text{NH}_4\text{Cl}$  were added.

Dewan [1938], in his paper on glutamic dehydrogenase, says that this enzyme is Co I-specific. The exps. of Fig. 11, however, make it clear, in addition to the previous results [Euler *et al.* 1938], that this is not the case. The curves show the hydrogenation of Co I by glutamic acid and apodehydrogenase from liver, and

the hydrogenation of Co II by the same system. When the equilibrium was nearly reached in the experiment with Co II, acetaldehyde was added, but had no effect; if, during the reaction, a conversion of Co II into  $\text{CoH}_2$  I had occurred, the extinction would have disappeared because the enzyme contained alcohol apodehydrogenase. However, after addition of ketoglutaric acid and  $\text{NH}_3$ , the equilibrium was pushed back and the  $\text{CoH}_2$  II formed was reoxidized.

Fig. 12 demonstrates the oxidoreduction between isocitric acid and iminoglutaric acid. In this experiment Co II was hydrogenated by isocitrate in presence of a dialysed aqueous extract from acetone-dried kidney, which contains highly active isocitric as well as glutamic dehydrogenase. Addition of  $\alpha$ -ketoglutaric acid alone had no effect, but when  $\text{NH}_3$  was added, the extinction fell off instantaneously, showing that the  $\text{CoH}_2$  II formed in the isocitric system was reoxidized by the iminoglutaric acid.

If no extra ketoglutaric acid had been added in this experiment the oxidoreduction would have occurred as well, because then the ketoglutaric acid formed from isocitric acid would have been aminated. But the concentration of iminoglutaric acid would have been so small that the rate of  $\text{CoH}_2$  II-disappearance would probably have been less than the rate of re-hydrogenation of the Co II by the excess of isocitric acid, and no decrease of extinction would have been observed until the whole of the isocitric acid had been used up. It is, however, possible to demonstrate the direct transformation of isocitric acid into glutamic acid (Fig. 13) if an excess of Co II is used instead of an excess of isocitric acid. Then, in the first phase of the experiment, the total amount of isocitric acid is

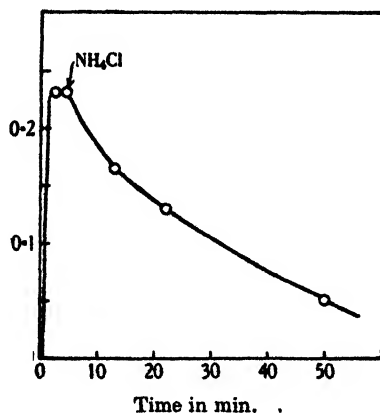


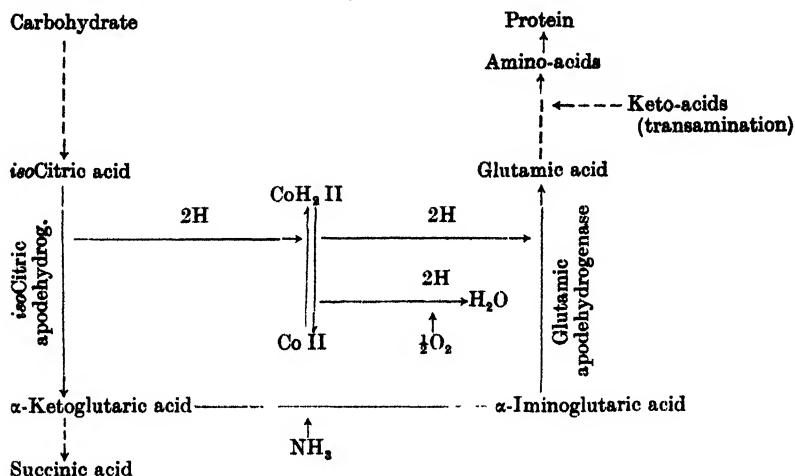
Fig. 13. Oxidoreduction between isocitric acid and iminoglutaric acid.  $1.8 \times 10^{-4} M$  *dl*-isocitric acid,  $0.95 \times 10^{-4} M$  Co II, 0.1 ml. "enzyme C", which contains glutamic besides isocitric apodehydrogenase.

converted into ketoglutaric acid and an equivalent amount of  $\text{CoH}_2$  II is formed. If now  $\text{NH}_4\text{Cl}$  is added, iminoglutaric acid is formed, which dehydrogenates the  $\text{CoH}_2$  II. "Enzyme C", which contains both apodehydrogenases, was used in this experiment.

#### DISCUSSION

The mechanism of the transformation of isocitric acid and the function of the isocitric dehydrogenase system as a link between carbohydrate breakdown and

protein synthesis, can, according to the results presented in this paper, be symbolized as follows:



The products of *isocitric acid* dehydrogenation, ketoglutaric acid and  $\text{CoH}_2 \text{ II}$ , can react further in two directions. Either they can be used in the glutamic dehydrogenase system for the fixation of  $\text{NH}_3$  (glutamic acid synthesis), or  $\text{H}$  can be transferred from the  $\text{CoH}_2 \text{ II}$  over diaphorase II and the cytochrome system to  $\text{O}_2$ , and the ketoglutaric acid can be broken down to succinic acid. The  $\text{H}$  necessary for the reductive amination of ketoglutaric acid can come, via  $\text{CoH}_2 \text{ I}$  or  $\text{CoH}_2 \text{ II}$ , from other dehydrogenase systems too, but it appears likely that the direct coupling of the *isocitric* and glutamic acid systems will be the most effective way. Ketoglutaric acid is regenerated from glutamic acid by transamination [Braunstein & Kritzmman, 1937].

According to the "citric acid cycle" theory of Krebs [cf. Krebs & Eggleston, 1938 and preceding papers] the system *isocitric acid*—ketoglutaric acid is part of a catalytic system in the oxidative breakdown of carbohydrate. If this theory is correct, codehydrogenase II is an indispensable part of the complex of cell respiration, especially the pyruvic acid oxidation, whilst codehydrogenase I is known to be necessary for the removal of the first pair of  $\text{H}$  atoms from the carbohydrate, i.e. from the triosephosphate molecule.  $\text{Mn}^{++}$ , as a complement of *isocitric* dehydrogenase, would also be involved in that part of cell respiration which concerns the breakdown of pyruvic acid. It seems possible that one could find support for the citric acid cycle theory by studying the action of  $\text{Co II}$  and  $\text{Co I}$  separately as well as of  $\text{Mn}^{++}$  on cell respiration.

#### SUMMARY

1. *isoCitric* apodehydrogenase was prepared from heart muscle. This enzyme catalyses specifically the dehydrogenation of *isocitric acid* by codehydrogenase II. The substrate affinity of the enzyme is extremely high. Citric acid is used only if aconitase is present in the enzyme preparation.
2. When the system was completed with flavinenzyme,  $1/2 \text{ mol. O}_2$  was taken up and  $1 \text{ mol. CO}_2$  was formed per mol. *isocitric acid*, and  $\alpha$ -ketoglutaric acid was isolated as the reaction product.

3. The system isocitric acid + Co II + apodehydrogenase does not react unless  $Mn^{++}$  or  $Mg^{++}$  are present;  $Mn^{++}$  is more active than  $Mg^{++}$ .

4. Iodoacetic acid and pyrophosphate are strong inhibitors, the first reacting with the apodehydrogenase, the second binding Mn and Mg ions.

5. isocitric apodehydrogenase was found in all animal tissues so far examined; the highest concentrations are present in heart, liver, kidney and adrenal gland.

6. The mechanism of the conversion of isocitric acid into glutamic acid was demonstrated and the biological importance of this reaction discussed.

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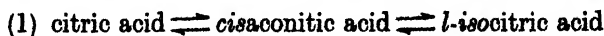
## CXXVII. ACONITASE<sup>1</sup>

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*(Received 1 May 1939)*

SINCE citric acid plays an important role in intermediary metabolism [Martius & Knoop, 1936; 1937; Krebs & Johnson, 1937] it was thought desirable to study in detail the reactions which citric acid may undergo in animal tissues. From the work of Martius & Knoop [1937] it is known that citric acid in the presence of tissue extracts forms an equilibrium with *cisaconitic acid* and *l-isocitric acid*:



The enzyme system bringing about the reversible hydration of *cisaconitic acid* has been shown to be different from fumarase and termed "aconitase" [Breusch, 1937]. This term will be used in this paper for the total enzyme system responsible for the reactions (1); it is left open whether one enzyme only brings about the two modes of reversible hydration of aconitic acid leading to citric acid or to *l-iso-citric acid*. The paper deals with some quantitative aspects of the reactions (1) especially with the investigation of the equilibrium between the three acids. After most of the work had been finished a paper of Martius appeared which partly covered the same ground [Martius, 1938]. The work reported in the present paper goes however beyond that of Martius in that it includes determinations of aconitic acid which Martius, lacking a suitable method, was unable to estimate.

### EXPERIMENTAL

#### *Preparation of cisaconitic acid*

Anhydro-acconitic acid was prepared from ordinary (*trans*) aconitic acid by the method of Anschütz & Bertram [1904]. Aconitic acid was boiled with acetyl chloride until a clear solution was obtained. The deep yellow solution was evaporated to dryness *in vacuo* over NaOH, and the brown solid was crystallized several times from hot benzene. Eventually a white solid was obtained, which melted sharply at 76°.

The benzene was removed by standing *in vacuo* over paraffin oil, and the solid stored in a desiccator over H<sub>2</sub>SO<sub>4</sub>.

*Cisaconitate* solution was freshly prepared before use; a known amount of anhydro-acconitic acid was dissolved in ice-cold water and neutralized with NaHCO<sub>3</sub>.

#### *Quantitative determination of citric acid*

The method of Pucher *et al.* [1936] was used. Citric acid is oxidized in the presence of bromine to pentabromoacetone which is subsequently converted into a coloured material by means of Na<sub>2</sub>S, and is estimated colorimetrically. The use of dioxan as the colour stabilizer in place of pyridine was found to be much less objectionable, and did not affect the accuracy of the method. If the amount of citric acid to be determined exceeded 1 mg. the depth of colour was not strictly

<sup>1</sup> Preliminary note in *Chem. & Ind.* 58, 56 (1939).

proportional. The solutions were therefore suitably diluted or else corrections were obtained from a calibration curve.

*Cis*aconitic acid did not give a colour when treated by this method.

#### *Quantitative determination of aconitic acid*

The method is based on the determination of the hydrogen required for the quantitative catalytic hydrogenation of the double bond. The catalyst used was a deposit of palladium on barium sulphate recommended by Köppen [1932], obtained from the Membranfilter G.m.b.h., Göttingen. The catalyst was suspended in water (10 mg./ml.) and a stream of  $H_2$  bubbled through. For the manometric determination the flasks were filled as follows:

	Main compartment	Side bulb
(1)	2 ml. water 1 ml. 5% $H_2SO_4$ 0.5 ml. Pd suspension	—
(2)	Do.	1 ml. test solution (acidified)

The manometers were filled with cylinder  $H_2$  washed with alkaline pyrogallol and acid dichromate. Rapid equilibration occurred at 40°, and in pure solution of aconitic acid the reaction was completed in 40 to 60 min. (see Table I).

Table I.  $H_2$  uptake with pure aconitic acid solution in presence of palladium catalyst. Conditions as above

Aconitic acid added = 172  $\mu$ l.

Time from mixing (min.)	15	30	50	70	290
Extra hydrogen uptake over "blank" ( $\mu$ l.)	106	153.5	170	171	170

In applying this method to the determination of aconitic acid in biological media difficulty was encountered owing to the interference of proteins with the catalyst. The choice of protein precipitant is limited to substances which are not themselves reducible. The method finally adopted was as follows. The solution to be tested was deproteinized by the addition of 1/5th volume of 5%  $HPO_3$  (freshly prepared). The precipitate was filtered off, and an aliquot part of the clear filtrate was strongly acidified with  $H_2SO_4$ . The aconitic acid was extracted 2 hr. with ether in a Kutscher-Steudel [1903] extractor. One ml. of water was added to the ethereal extract and the ether evaporated off. The residue was made up to a known volume with 5%  $H_2SO_4$ , and the final solution tested as above.

The partition coefficient of *cis*aconitic acid between ether and water was found to be about 15:1 in favour of the aqueous phase at 20°. Under our conditions complete recovery was obtained with 2 hr. extraction.

#### *Recovery of aconitic acid added to extract of pigeon breast muscle (dilution 1 in 5)*

Mixtures were made up with varying amounts of aconitic acid as follows:

10 ml. extract + 1 ml. 5%  $H_2SO_4$  + 4 ml. 5%  $HPO_3$  + aconitic acid.

The recovery of aconitic acid after treatment as above is given in Table II.

The reaction is general for a double bond and works equally well with *cis*- and *trans*-aconitic acids, crotonic and fumaric acids. The latter can be estimated by re-extracting after hydrogenation and determining the succinic acid formed by the method of Szent-Györgyi as modified by Krebs [1937].



Table II. *Recovery of aconitic acid from pigeon breast muscle extract*

No.	Aconitic acid added ( $\mu$ l.)	H <sub>2</sub> uptake ( $\mu$ l.)	Aconitic acid recovered	% recovery
1	—	116	—	—
2	1255	1320	1204	96
3	628	695	579	92
4	251	355	239	95

Hydroxy-acids do not react under these conditions, and ketonic acids react only slowly. As the addition of citrate to the dilute tissue extracts used does not give rise to the formation of ketonic acids under the conditions of our experiments, the method is sufficiently specific for our purpose.

#### *Metabolic quotients*

In most experiments the rates of reaction are expressed by the quotient

$$\frac{\text{micromol. of substrate metabolized}}{\text{mg. tissue} \times \text{hours}}.$$

In some experiments it seemed desirable to compare the rates of citric acid breakdown or formation with the rate of O<sub>2</sub> uptake, since citric acid plays a role in the oxidative metabolism. The quantities of citric acid metabolized are therefore expressed in some tables in  $\mu$ l., 1 m.-mol. citric acid being considered equivalent to 22,400 $\mu$ l. The rate of the metabolic reaction is expressed by the quotient

$$\frac{\mu\text{l. substrate metabolized}}{\text{mg. dry tissue} \times \text{hours}}.$$

#### *The reaction cisaconitic acid $\rightarrow$ citric acid*

*Extraction of "aconitase".* The majority of experiments were performed with extracts of pigeon breast muscle. Chilled breast muscle was minced through a Latapie mincer and ground with 5 vol. 0.1 M phosphate buffer of pH 7.4 (except where otherwise stated). Quartz (Merck's quartz sand "washed and calcined") was used to break up the tissue. The resulting suspension was rapidly centrifuged and the supernatant liquid used. This will be referred to as the "stock enzyme solution".

Aconitase is almost completely extracted by this method; a second or third extraction shows only traces of activity, as illustrated by the following experiment.

Table III. *Extraction of aconitase from muscle tissue*

1 ml. stock enzyme, 2 ml. phosphate buffer pH 7.4, 1 ml. cisaconitate (0.0081 M). 40°.

	Citric acid formed after 30 min. incubation $\mu$ mol.
First extract	6.8
Second extract	0.9
Third extract	0.27
Fourth extract	0

The activity of the enzyme does not deteriorate within a fortnight if kept in the refrigerator.

*The course of the citric acid formation from cisaconitic acid.* To follow the rate of formation of citric acid from cisaconitic acid it was convenient to use dilute extracts, since the reaction is very rapid in the stock solution. Stock enzyme solution from pigeon breast muscle was diluted 50 times with 0.1 M phosphate

buffer (pH 7.4). 3 ml. dilute extract were incubated with 1 ml. *cisaconitate* (0.00735 *M*) at 40° under anaerobic conditions, and the formation of citrate was measured at various intervals.

Table IV

Time of incubation (min.)	10	20	40	60	120
Citrate formed (μmol.)	2.2	4.1	5.6	6.0	6.4

The initial rate of citrate formation, expressed in the terms of a metabolic quotient, is thus:

$$Q_{\text{citrate}} = 120 \mu\text{l./mg./hr.}$$

*The position of equilibrium cisaconitic acid  $\rightleftharpoons$  citric acid in muscle extract.* In the previous experiment the final amount of citrate represents 87% of the added *cisaconitate*. The following table shows that in other experiments in which the reaction was allowed to reach the equilibrium, the reaction generally stopped when 75–85% of the added *cisaconitate* had been converted into citrate.

Table V. Stock enzyme solution diluted with 0.1 *M* phosphate buffer pH 7.4. 40°. *N*<sub>2</sub>

No.	Dilution of stock enzyme solution	Time of incubation min.	<i>Cisaconitate</i> added μmol.	Citrate formed μmol.	% conversion
1	3	130	7.9	6.75	85
2	9	60	7.0	5.2	75
3	40	60	6.35	5.35	85
4	10	120	7.55	5.83	78
5	2	120	6.85	5.35	78

*The pH optimum of aconitase in muscle extract.* The experiments were arranged in such a way as to allow the determination of the initial rates of citric acid formation from *cisaconitic acid* by extracts of different pH. The curve shows a sharp maximum at pH 7.4. It will later be seen that the (apparent) optimum in tissue slices is at 7.9.

Table VI. pH optimum

3 ml. enzyme (stock enzyme diluted 60 times with buffer of appropriate pH) + 1 ml. *cisaconitate* (0.01 *M*). 20 min. at 40°

pH	2.6	3.9	4.9	6.2	6.8	7.4	8.5	9.2
Citrate formed (μmol.)	0.27	0.2	0.7	1.2	4.1	5.0	2.8	1.5

*Activity of other tissues. Intact cells.* Aconitase occurs in all the animal tissues which we examined, with the exception of red blood cells. The rate of formation of citric acid from *cisaconitic acid* in various tissues is given in the following table:

Table VII. Citric acid formation from *cisaconitic acid* by rat tissues

Bicarbonate saline. 5% CO<sub>2</sub> in *N*<sub>2</sub>. 40°. Initial concentration of *cis-aconitate* = 0.025 *M*.

Tissue	Time of shaking min.	$Q_{\text{citrate}}$ μl./mg./hr.
Liver	30	8.0
(sliced)	60	8.5
Kidney cortex	30	12.5
(sliced)	60	14.0
Testis	30	4.2
(teased)	60	5.0
Brain cortex (sliced)	60	4.6

The rate increases on raising the initial concentration of *cisaconitic acid* (Table VIII).

Table VIII. *Citric acid formation from varying concentrations of cisaconitic acid by rat kidney cortex slices*

Bicarbonate saline. 60 min. at 40°. 5 % CO<sub>2</sub> in N<sub>2</sub>.

Initial concentration <i>cisaconitate</i>	0.0033 <i>M</i>	0.014 <i>M</i>	0.025 <i>M</i>
<i>Q</i> <sub>citrate</sub>	5.9	16.9	21.7

*Tissue extracts.* Extracts of rat tissues were made in the same way as described for pigeon breast muscle, except that the preliminary mincing was omitted. In extracts the rate of reaction was always much higher than in slices.

Table IX. *Citric acid formation from cisaconitic acid by rat tissue extracts*

Phosphate buffer pH 7.4. N<sub>2</sub>. 60 min. at 40°. Initial concentration *cisaconitate* = 0.025 *M*.

Tissue extracted	<i>Q</i> <sub>citrate</sub> μl./mg./hr.
Kidney cortex	80.0
Liver	61.5
Lung	14.5
Submaxillary glands	11.9
Whole brain	10.0
Intestine	7.8
Testis	7.6
Red blood cells	0
Pigeon breast muscle	120.0

The slower rate of reaction observed in slices may be due to the fact that the pH in the tissue is not optimal for the reaction. It has been shown that aconitase in pigeon breast muscle extract shows a sharp pH maximum at 7.4. The following experiment shows that the pH optimum is different in slices (about pH 8.0).

Table X. *Formation of citric acid from cisaconitic acid by rat liver slices in bicarbonate medium of varying pH*

Bicarbonate saline. CO<sub>2</sub> in N<sub>2</sub>. 30 min. at 40°. Initial concentration *cisaconitate* = 0.067 *M*.

pH	8.3	8.0	7.8	7.65
<i>Q</i> <sub>citrate</sub>	54.2	75.5	71.0	55.0

It is probable that this difference is an apparent one. The concentration of bicarbonate in the tissue is known to be lower than in the medium, whilst the concentrations of free CO<sub>2</sub> are of the same order in medium and tissue. The pH of the tissue is thus lower than that of the medium. The experiment recorded in Table X does not therefore represent the real pH activity curve of aconitase. The latter can only be measured in extracts.

#### *The reaction citric acid → cisaconitic acid*

To measure the formation of *cisaconitic acid* from citric acid, pigeon breast muscle extract was incubated anaerobically at 40° with citric acid, and the resulting solution tested for the formation of aconitic acid. A small but definite quantity of aconitic acid, amounting finally to 4–5 % of the added citric acid, was found; this value did not further increase on prolonged incubation.

Table XI. *Course of aconitic acid formation from citric acid*

Stock enzyme solution (diluted 12 times) in phosphate buffer pH 7.4. 0.02 M citrate. 40°.

Time of shaking (min.)	5	10	20	40
Aconitate formed ( $\mu$ mol.)	2.8	4.0	4.8	5.9

Citric acid added = 146  $\mu$ mol.

The effect of the citric acid concentration on the formation of aconitic acid is shown in the following table:

Table XII. *Formation of aconitic acid from citric acid (final values; effect of citrate concentration)*

Pigeon breast muscle extract. pH 7.4. 40°.

Citrate added $\mu$ mol.	Aconitate formed $\mu$ mol.	% conversion
145	5.9	4.1
5020	240.0	4.8
2480	127.0	5.1
3380	150.0	4.4
4055	193.0	4.75
1000	42.0	4.2

*Formation of isocitric acid*

The following tables give the results of experiments in which the sum of the citric acid and aconitic acid remaining after incubation was compared with the amount of substrate added.

Table XIII. *Citric acid formed and aconitic acid remaining after incubation of pigeon breast muscle extract with cisaconitic acid*

Aconitate added $\mu$ mol.	Time of incubation min.	Citrate formed $\mu$ mol.	Aconitate left $\mu$ mol.	Loss	% loss
754	5	87	486	181	24
754	30	450	104	100	13
754	120	582	65	107	14
685	120	536	60	89	13
1020	90	615	285	140	14

Table XIV. *Aconitic acid formed and citric acid left, after incubation of pigeon breast muscle extract with citric acid*

Citrate added $\mu$ mol.	Time of incubation min.	Aconitate formed $\mu$ mol.	Citrate left $\mu$ mol.	Loss	% loss
2480	30	108	1680	692	28
2480	120	127	1790	563	22
3380	330	150	2780	450	13
4050	1320	193	3150	707	17
1000	90	46	830	124	12

In all experiments, the measured sum of citric and aconitic acid equivalents is less than the amount of substrate added. This indicates the formation of a third substance. From schemes put forward previously [Martius, 1937] the missing fraction was expected to be isocitric acid, and experiments were performed in which the development of optical rotation in the extracts after incubation with either citric or cisaconitic acids was measured. These experiments will not be reported in detail since they do not go beyond Martius's results [1938].

In accordance with Martius the rotation observed corresponded to 10–20% *isocitric acid*, in agreement with the view that the above mentioned deficit is due to the formation of *isocitric acid*. Our experiments also confirm Martius's finding of a rapid formation of *isocitric acid* from *cisaconitic acid*, followed by a partial disappearance (see curve 2 of Martius's paper).

*Aconitase in cucumber seeds*

Aconitase also occurs in cucumber seeds [Martius, 1938] and it seemed of interest to compare the enzymes from different sources. An extract of dried cucumber seeds was prepared according to the directions of Scherstén [1936]. One part of ground seed kernel was extracted with two parts 0.87%  $K_2HPO_4$  and the centrifuged extract used.

(a) *Incubation with cisaconitic acid*. The amount of citric acid formed by incubation of this extract with *cisaconitic acid*, together with the amount of aconitic acid remaining in the solution, was measured. Table XV summarizes the results of three experiments.

Table XV. *Cucumber seed extract incubated with cisaconitate 40°. Octyl alcohol added*

Initial concentration of aconitic acid = 0.03 M approx.

No.	Vol. of enzyme ml.	Aconitate added $\mu$ mol.	Citrate formed $\mu$ mol.	Aconitate left $\mu$ mol.	Loss	Time of incubation min.
1	15	500	237 (47%)	74.5	191 (38%)	120
2	15	607	58 (9%)	290	259 (42%)	20
		607	121 (20%)	250	235 (39%)	40
		607	168 (27%)	220	218 (36%)	60
		607	231 (27%)	177	198 (32%)	120
		607	295 (48%)	99	214 (35%)	240
3	15	540	87 (16%)	300	155 (29%)	30

(b) *Incubation with citric acid*. In another series of experiments cucumber seed extract was incubated with citric acid, and the amount of aconitic acid formed, together with the amount of citric acid remaining, was determined (Table XVI).

Table XVI. *Cucumber seed extract incubated with citrate at 40°. Octyl alcohol added*

Initial concentration of citrate =  $M/30$ . Amounts of metabolites given in  $\mu$ mol.

No.	Vol. of enzyme ml.	Citrate added	Aconitate formed	Citrate left	Loss	Time of incubation min.
1	15	500	21 (4.2%)	466	13 (2.6%)	30
2	15	500	23 (4.6%)	428	49 (5.8%)	60
3	15	500	22 (4.4%)	500	—	120
4	15	500	29 (5.8%)	476	—	240
5	15	500	27 (5.4%)	466	9 (1.8%)	360

From these figures it is seen that the activity of the enzyme in cucumber seed is very much lower than in muscle (compare Table IV and Tables XV and XVI). The initial velocity of the reaction in cucumber seed extract is about 1.6% of that in muscle extract. Both reactions, *cisaconitic acid*  $\rightarrow$  citric acid and citric acid  $\rightarrow$  *cisaconitic acid* take place in cucumber seed extract, but no true equilibrium was reached in our experiments.

There is a small deficit when citric acid is the substrate (2–6%) but a very considerable loss when *cis*aconitic acid is the substrate (32–42%). The fact that no equilibrium is established may be due to the comparatively low activity of the enzyme.

#### DISCUSSION

Aconitase is an unusual enzymic system in that it forms two different isomeric compounds through addition of water to a double bond. No analogy is known to occur amongst enzymes, but many similar reactions are known to the organic chemist. For example, the addition of iodine monochloride to propylene gives  $\text{CH}_3\text{CHCl}.\text{CH}_2\text{I}$  (69%) and  $\text{CH}_3\text{CHI}.\text{CH}_2\text{Cl}$  (31%) [Ingold & Smith, 1931]. These reactions, however, are not reversible.

#### SUMMARY

1. A method has been worked out for the quantitative determination of aconitic acid based on the catalytic hydrogenation of the double bond.

2. The action of aconitase (the conversion of citric acid into *cis*aconitic acid and the reverse reaction) has been studied in animal tissues. Aconitase is extracted with *M*/10 phosphate buffer pH 7.4 from minced pigeon breast muscle and other tissues.

3. In tissue extracts aconitase converts about 75–85% of added *cis*aconitic acid into citric acid.

4. Citric acid incubated with pigeon breast muscle extract forms about 4% aconitic acid.

5. The equilibrium is reached when the solution contains about 16% *iso*-citric acid (Martius), about 4% *cis*aconitic acid and about 80% citric acid.

6. The pH optimum of aconitase in tissue extracts is at 7.4. In tissue slices the (apparent) optimum is 7.9.

7. Aconitase was found in pigeon breast muscle, rat liver, kidney cortex, testis, brain cortex, lung, submaxillary gland, intestine, but not in red blood cells of the rat.

8. Aconitase also occurs in cucumber seeds but its activity is only 1.6% of that of pigeon breast muscle and no true equilibrium between *cis*aconitic, *iso*-citric and citric acids was reached with cucumber seed extract.

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